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The Autolysis of Human HtrA1 Is Governed by the Redox State of Its N-Terminal Domain

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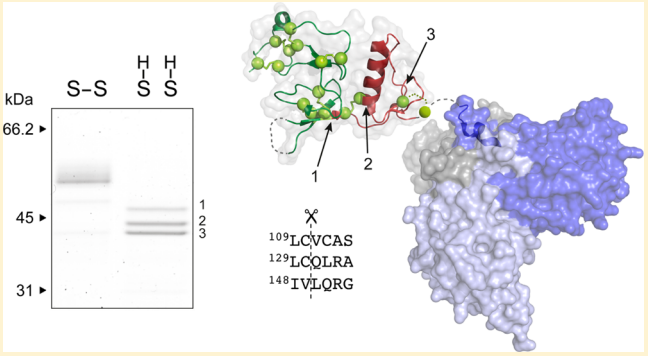
ABSTRACT: Human HtrA1 (high-temperature requirement protein A1) belongs to a conserved family of serine proteases involved in protein quality control and cell fate. The homotrimeric ubiquitously expressed protease has chymotrypsin-like specificity and primarily targets hydrophobic stretches in selected or misfolded substrate proteins. In addition, the enzyme is capable of exerting autolytic activity by removing the N-terminal insulin-like growth factor binding protein (IGFBP)/Kazal-like tandem motif without affecting the protease activity. In this study, we have addressed the mechanism governing the autolytic activity and find that it depends on the integrity of the disulfide bonds in the N-terminal IGFBP/Kazal-like domain. The specificity of the autolytic cleavage reveals a strong preference for cysteine in the P1 position of HtrA1, explaining the lack of autolysis prior to disulfide reduction. Significantly, the disulfides were reduced by thioredoxin, suggesting that autolysis of HtrA1 *in vivo* is linked to the endogenous redox balance and that the N-terminal domain acts as a redox-sensing switch.



Human HtrA1 (high-temperature requirement protein A1, Uniprot entry Q92743) is a 51 kDa nonglycosylated serine protease primarily located in the extracellular space. Here, it is involved in remodeling of the extracellular matrix by interactions and proteolysis of components like decorin, fibronectin, aggrecan, and collagen.[1−5] The protease also regulates cellular signaling cascades by specific breakdown of mediators like transforming growth factor-β, fibroblast growth factor 8, and epidermal growth factor receptor.[6−9] In addition, HtrA1 is colocalized in the cytoplasmic compartment with several intracellular identified substrates, such as tubulin and the X-linked inhibitor of apoptosis (XIAP).[10] Dysregulation of HtrA1 expression levels is associated with cerebral small-vessel disease,[11] age-related macular degeneration (AMD),[12,13] osteoarthritis,[14] amyloid neurological disorders,[15,16] corneal dystrophy,[17] preeclampsia,[18,19] and numerous types of cancer.[20,21] The specific downregulation of HtrA1 in the latter case promotes the sustained survival of cancer cells and the development of malignant metastatic behavior,[20,22,23] most likely correlated with the intracellular HtrA1 associated with microtubules and affecting cell migratory and signaling properties.[3,14,24−27] Interestingly, autolytically N-terminally truncated forms of HtrA1 exist *in vivo*, and such forms have been suggested as prognostic markers in urothelial bladder cancer[28] and in preeclamptic pregnancies.[18] The exact role and trigger of autolytic maturation is at present unknown.

HtrA1 is composed of three domains; the central core contains the catalytic triad (His[220], Asp[250], and Ser[328]) and is responsible for enzymatic activity and most reported functions, such as substrate specificity, proteolysis, trimerization, and liposome and cell-surface interactions.[29] The N- and C-terminal domains of HtrA1 comprise an IGFBP-like/Kazal-like tandem domain motif (termed the N-domain) and a PDZ domain, respectively. Both seem largely dispensable for catalytic function,[30] and whereas the PDZ domain facilitates protein−protein interactions by tethering of the HtrA1 protease to specific sites in the ECM or in the cell,[4,26,31] the role of the N-domain remains enigmatic. The domain contains a total of 16 cysteines, all forming part of an intensive disulfide bond network (Figure 1). It has previously been shown that HtrA1 may undergo autolytic cleavage, and it was therefore suggested that the N-terminal Kazal-like subdomain could play an autoinhibitory function in this context.[14] However, recent studies have shown that neither the IGFBP- nor the Kazal-like structures have retained their canonical functions required for IGF interaction or protease inhibition, respectively.[30] The lack of autoinhibition by the N-domain is further corroborated by studies showing similar catalytic activities of full-length HtrA1 and ΔN-HtrA1 lacking the N-domain.[11,30]

Received: December 6, 2013
Revised: May 20, 2014
Published: May 20, 2014

 © 2014 American Chemical Society dx.doi.org/10.1021/bi401633w | *Biochemistry* 2014, 53, 3851−3857

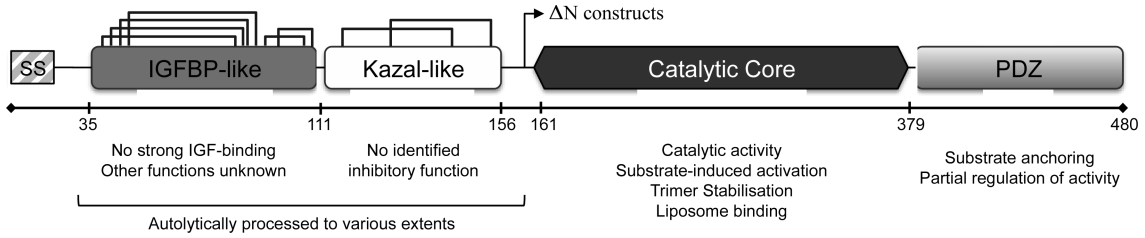


Figure 1. HtrA1 domain organization and disulfide bond network.

The presence of processed HtrA1 forms in the intracellular reducing environment suggests that autolysis could be affected by changes in the redox state of HtrA1, more specifically the integrity of the N-domain disulfide bonds. In this study, we investigate the effect of antioxidant components dithiothreitol (DTT), reduced glutathione (GSH), and thioredoxin (TRX) on the activation of autolytic processing of full-length HtrA1. We show that the TRX redox regulator is a physiologically relevant trigger of autolysis and identify the major autolytic forms, which are in good correlation with previous observations. In addition, the autolytic activity displays a remarkably strong preference for cysteine in the P1 position, which is novel insight into the action and specificity of HtrA1 and explains the sensitivity toward the redox state of the surrounding environment. Thus, we speculate that the autolysis of HtrA1 and the presence of various mature forms *in vivo* could be correlated to disease-related dysregulations in the endogenous redox balance.

MATERIALS AND METHODS

Cloning and Expression. HtrA1 cDNA was obtained from Invitrogen and amplified to incorporate a C-terminal His tag separated from the C-terminus of HtrA1 by a FX protease cleavage site. The construct was cloned into the pcDNAFRT/TO vector (Invitrogen) using KpnI and XhoI restriction sites. The resulting mature translated protein (theoretical molecular mass of 50.3 kDa) is termed HtrA1. Production of HtrA1 was achieved in Freestyle 293-F cells (Life technologies) transfected with the FRT-HtrA1-His plasmid at a cell density of 1×10^6 cells/mL using polyethyleneimine (PEI). The culture supernatant was collected 48 h after transfection and dialyzed overnight at 4 °C into 20 mM Tris-HCl and 300 mM NaCl (pH 8.0). The HtrA1 protein was purified by Ni affinity chromatography using a 5 mL Hitrap Chelating column (GE Healthcare Life Sciences). The imidazole-eluted fractions were pooled and dialyzed into TBS {Tris-buffered saline [20 mM Tris-HCl and 150 mM NaCl (pH 7.4)]}. The purity and concentration was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The N-terminal amino acid sequence of the 50 kDa HtrA1 band was determined to be Ser^{30}-Ala-Pro by Edman degradation, in agreement with previous findings.[32]

HtrA1 Reduction and Autolytic Cleavage. Reaction mixtures for testing HtrA1 disulfide reducibility were set up with 65 μL of TBS containing 1.5 μg of phenylmethanesulfonyl fluoride (PMSF)-treated HtrA1 (2 mM PMSF for 2 h) and increasing concentrations of reducing agents DTT, GSH, and TRX, all from Sigma-Aldrich. Reaction mixtures were incubated at 37 °C for 30 min (DTT) or 1 h (GSH and TRX). The TRX concentration range was selected to reach a 1:1 ratio of the total number of cysteine bridges to TRX molecules at 4 μM TRX. After incubation, samples were then treated for 10 min with 15 mM iodoacetamide (IAA) to block free cysteines and to scavenge the excess reducing agents present in the reaction mixture before SDS–PAGE. For the assessment of autolytic cleavage in the presence of reducing agents, reaction mixtures with catalytically active HtrA1 (1.5 μg) were set up with either 1 mM DTT, 1 mM GSH, or 4 μM TRX and incubated for 20 h at 37 °C. The activity was tested by titrating HtrA1 (full-length or the autolytic products) against a fixed amount of β-casein (3 μg). The proteolytic β-casein products were analyzed after incubation for 30 min at 37 °C by SDS–PAGE.

SDS–PAGE and Edman Degradation. Samples were boiled for 5 min in SDS–PAGE sample buffer in the presence or absence of reducing agents, as specified in the text and figure legends. Proteins were separated in 8 or 10 to 15% (w/v) gradient polyacrylamide gels and run using the discontinuous ammediol/glycine buffer system.[33] Gels were stained with Coomassie Brilliant Blue or blotted for 20 min to an Immobilon-P membrane (Millipore) for N-terminal protein sequence analysis using a Procise 494-HT protein sequencer (Applied Biosystems).

Analysis of Autolytic Peptides by Mass Spectrometry. HtrA1 (0.5 μg, 20 μg/mL) was incubated at 37 °C in the presence or absence of 5 mM DTT for 3 or 20 h. The reaction was quenched by the addition of formic acid to a final concentration of 1%. The samples, incubated for 20 h, were pretreated with 10 mM fresh DTT for 15 min to ensure complete reduction of the disulfides before the formic acid quenching step. Then they were micropurified using C18 stage tips (Thermo Fisher Scientific), dried down in a SpeedVac, and dissolved in 0.1% formic acid for liquid chromatography–tandem mass spectrometry analysis. The samples were run three consecutive times on an AB Sciex TripleTOF 5600 instrument. Data analysis was conducted using the Mascot search engine with a peptide fragment mass tolerance of 0.2 Da. Only peptides with a significance score of <0.01 and an ion score cutoff of >30 were considered reliable hits and included in the final list of identified HtrA1 N-terminal peptides. For an autolytic cut site to be valid, the given P1 residue had to be identified in at least two of the three replicates. MS Data Miner[34] was used for the extraction of relevant data from the Mascot search file.

RESULTS

Reducing Agents Differentially Affect HtrA1 Disulfide Bridges. Full-length HtrA1 was expressed in the Freestyle 293 human cell line and purified by Ni-NTA chromatography. To explore the stability of the disulfides in the N-domain, we analyzed the susceptibility of PMSF-inhibited HtrA1 to reduction by DTT, GSH, or TRX followed by nonreducing SDS–PAGE (Figure 2). DTT and TRX but not GSH effectively led to a concentration-dependent HtrA1 reduction, which was evident in the disappearance of the oxidized HtrA1 band (Figure 2). The reduction of HtrA1 happened gradually

dx.doi.org/10.1021/bi401633w | *Biochemistry* 2014, 53, 3851–3857

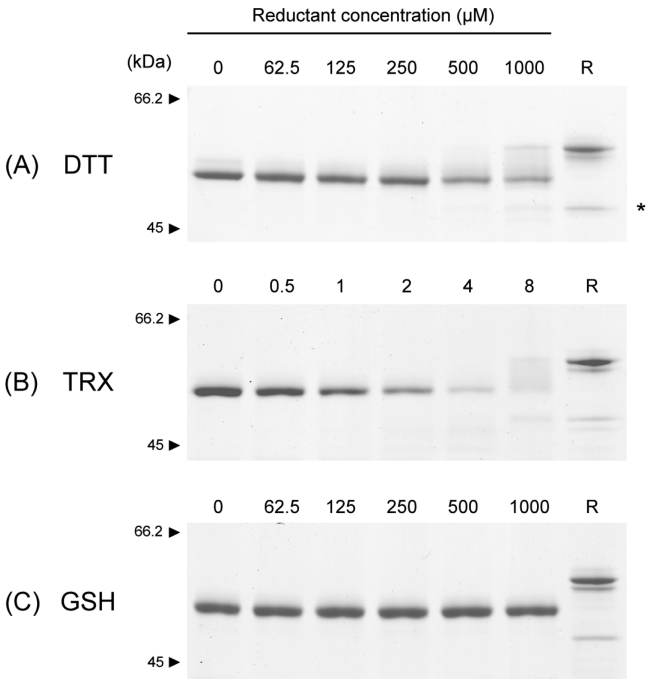


Figure 2. Stability of HtrA1 disulfide bonds toward reducing agents. HtrA1 (1.5 μg in each lane) was incubated with freshly prepared reducing agents at the indicated concentrations for either 30 min (DTT) or 1 h (TRX and GSH) at 37 °C. Samples were then alkylated with IAA and analyzed by nonreducing SDS−PAGE using 8% gels. The reduction of HtrA1 by the different agents is reflected by the disappearance of protein from the fully oxidized band. For 4 μM TRX reduction, large cross-linked species were present at the top of the gel, representing reoxidized intermolecular disulfides and thereby explaining the apparent lack of protein at the expected sizes. A reduced control sample (R) was boiled in the presence of 5 mM DTT and subsequently alkylated with IAA before gel electrophoresis. The asterisk indicates a lower-molecular mass form of HtrA1 that is released upon reduction, representing a portion of HtrA1 that is nicked at loop residue Arg-103 or Arg-104 during cellular production and purification. The reduction of the disulfide bridges in HtrA1 is effectively achieved by DTT and TRX but not by GSH.

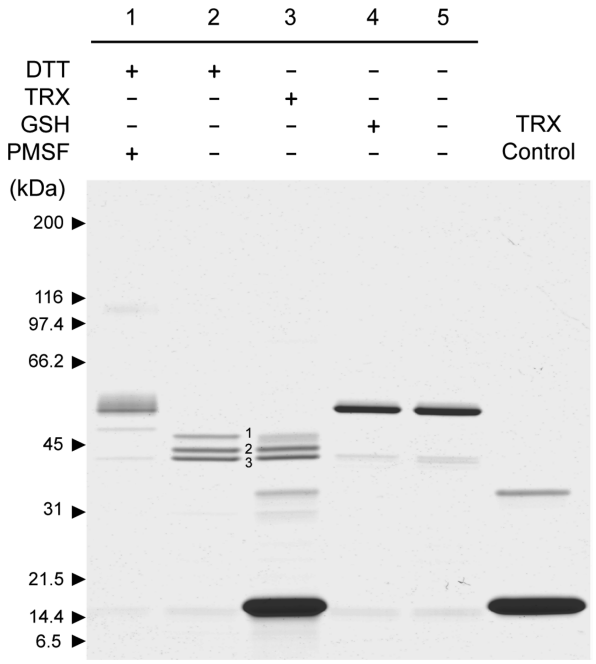


Figure 3. Disulfide bond reduction triggers HTRA1 autolysis. To active HtrA1 (1.5 μg) was added a reducing agent (1 mM DTT, 1 mM GSH, or 4 μM TRX) and the mixture was left for 20 h at 37 °C and subsequently analyzed by nonreducing SDS−PAGE using a 5 to 15% gradient gel. Controls were included for the effect of HtrA1 activity (lane 1, PMSF-treated) and for the effect of the reducing agent (lane 5, unreduced HtrA1). TRX alone was included for comparison (lane 6). The reduction of disulfide bridges led to effective HtrA1 autolysis when proteolytic activity was not inhibited, resulting in three major autolytic products for DTT- and TRX-treated samples. The presence of a smear in lane 1 and a faint high-molecular mass band around 100 kDa (dimeric species) was caused by reoxidation of disulfide bridges by a lack of reducing power as a consequence of DTT oxidation over time.

as a smear between the fully oxidized band (Figure 2A lane "0") and the fully reduced control (Figure 2A, lane "R") was clearly visible with increasing concentration of reductant. Higher-molecular mass HtrA1 bands were also observed, most likely because of reoxidation and formation of intermolecular disulfides during the incubation (not shown). This was more pronounced following the incubation with TRX, where the concentration of the oxidant was lower. The experiment showed that TRX was able to reduce the disulfide bridges in the N-domain of HtrA1. This has previously been shown to occur in other proteins by an intramolecular disulfide exchange cascade,[35] and a similar mechanism could be utilized during the TRX-mediated reduction of HtrA1.

Autolysis Correlates with the Reduction of Disulfide Bonds. The consequence of disulfide bridge reduction was investigated by incubating active HtrA1 for 20 h in the presence of 1 mM DTT, 1 mM GSH, or 4 μM TRX and analyzing the reaction products by SDS−PAGE (Figure 3). The analysis revealed three major bands with lower molecular masses when the incubation was conducted in the presence of DTT or TRX. GSH did not display noticeable differences with respect to the control; however, if the concentration was increased to 10 mM GSH, a small fraction of HtrA1 was also found to be processed to the same three major bands (data not shown). The proteolytic activity of the truncated forms was similar to that of intact HtrA1 as judged by the ability to hydrolyze β-casein (Figure 4). This was in support of a neglectable effect of the N-domain on protease activity. The three autolytic forms were then subjected to Edman degradation to determine the new N-termini resulting from the major autolytic cuts. These were found to be Val-111 (top band, cleavage site LC↓VC), Gln-131 (middle band, cleavage site LC↓QL), and Leu-150 (lower band, cleavage site IV↓LQ), resulting in theoretical molecular masses of 41.5, 39.5, and 37.2 kDa, respectively. While the 37.2 kDa form contained Cys-155, the two larger forms contained several cysteines. The presence of three bands indicated that autolysis was incomplete, and we speculated that reoxidation of cysteine residues during the 20 h incubation was hampering autolysis. This was investigated by incubating HtrA1 for 72 h in the presence of DTT (5 mM) with the addition of fresh DTT (5 mM) after 24 and 48 h. The results showed that HtrA1 autolysis under these conditions proceeded to the 37.2 kDa band, confirming the need for reduced cysteines in the process and explaining the lack of autolysis prior to disulfide reduction (Figure 5).

Autolytic Cleavage Sites Reveal P1 Specificity for Cysteine. The identification of the major autolytic cut sites indicated that HtrA1 cleaves efficiently with a cysteine in the P1 position. This specificity had not previously been described for

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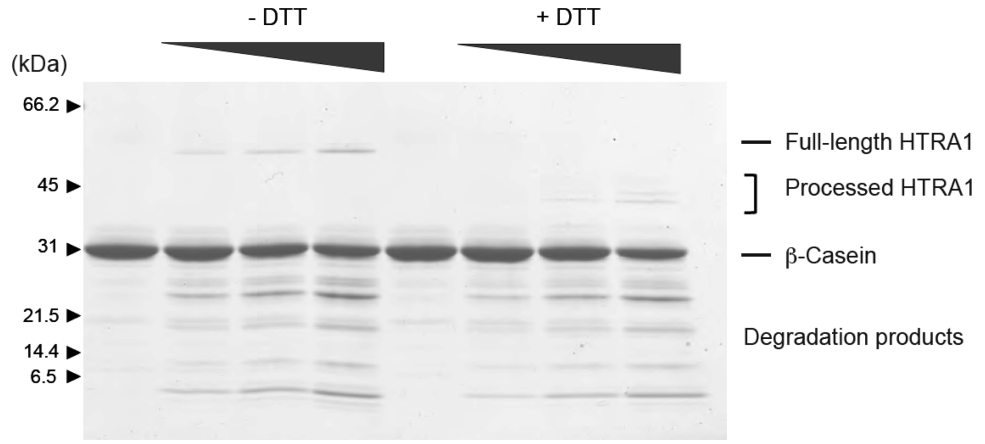

Figure 4. Autolytic forms display activity similar to that of full-length HtrA1. The activity of full-length HtrA1 and its autolytic products was tested by incubating β-casein (3 μg) with increasing amounts of HtrA1 (preincubated for 20 h with or without DTT). After 30 min, degradation products of β-casein were easily identified, and no significant difference in the intensity or sizes of the products was observed between full-length HtrA1 and the autolytically processed versions. Assays were also performed after incubation of β-casein with HtrA1 for 45 min or 1 h, giving similar results (data not shown).

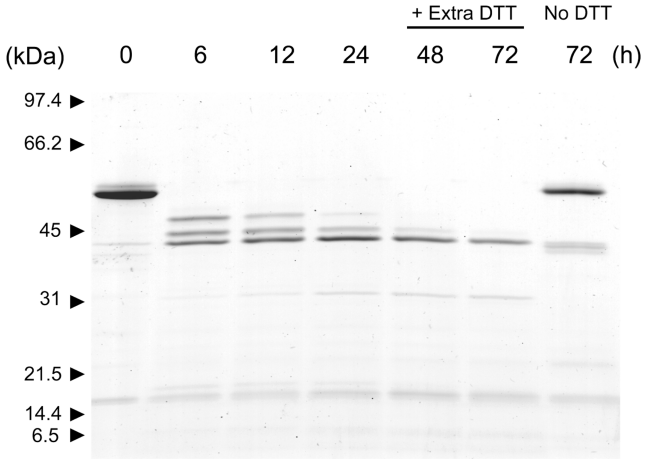

Figure 5. Continued reduction and incubation provides full HtrA1 autolysis. HtrA1 was incubated with 5 mM DTT, and 1.5 μg aliquots were removed at indicated time points, treated with 15 mM IAA for 15 min, and then boiled in SDS–PAGE sample buffer to block further autolysis. Additional 5 mM DTT was added after 24 and 48 h, providing fresh reducing power. Unreduced HtrA1 was included for comparison after incubation for 72 h. The continued HtrA1 autolysis in the presence of sufficient reducing power led to more complete trimming to the smallest of the three major autolytic forms.

HtrA1, and to gain further insight into HtrA1 autolytic P1 preferences, we used mass spectrometry to analyze the N-domain (residues 30–157) fragment peptide space generated after incubation with DTT for 3 or 12 h. The weighted frequency of the P1 occurrences from Ser-30 to Gln-157 is presented in Table 1. Indeed, cysteine was found to be one of the preferred cleavage sites with cleavage at 7 of 16 positions in the N-domain. The other identified P1 cleavage sites aligned well with previous reports on HtrA1 specificity.[29] Evidently, many autolytic peptides had already been generated after incubation for 3 h, suggesting rapid activation of autolysis upon disulfide bridge reduction. The central role of cysteines in this process probed us to investigate if general reduction of disulfide bridges in other cysteine-rich proteins facilitated proteolysis by HtrA1; however, no significant cleavage of reduced bovine pancreatic trypsin inhibitor (8 cysteines) or reduced human serum albumin (34 cysteines) was seen after incubation at 37 °C for 3 h (data not shown). This could indicate that autolysis by design is an inherent feature of HtrA1 controlled by its cysteine specificity.

Table 1. Unique Autolytic Cuts in the N-Terminal Domain Observed by Mass Spectrometry[a]

		no. of unique cuts		weighted frequency	
type (P1)	total no. in the N-domain	incubation for 3 h at 37 °C with 5 mM DTT	incubation for 20 h at 37 °C with 5 mM DTT	incubation for 3 h at 37 °C with 5 mM DTT	incubation for 20 h at 37 °C with 5 mM DTT
C	16	6	7	0.38	0.44
A	19	6	4	0.32	0.21
L	8	4	2	0.50	0.25
Q	7	2	2	0.28	0.29
R	13	2	2	0.15	0.15
T	2	1	1	0.50	0.50
V	9	1	1	0.11	0.11

[a]The position of each autolytic cut was identified by analysis of the generated peptides, and the unique cuts refer to the P1 residue type, looking exclusively at the N-domain (residues 30–157). The weighted frequency = (the number of unique cuts)/(total number in the N-domain).

DISCUSSION

Autolysis of HtrA family members has been observed previously, but the functional consequences and triggers of autolysis have been described only to a very limited extent. This study shows that HtrA1 autolysis is linked to the integrity of the disulfide bonds in the N-domain. Following reducing conditions, three major bands were observed via SDS–PAGE, corresponding to fragments starting at Val-111 (top band), Gln-131 (middle band), and Leu-150 (lower band) (Figure 3), in agreement with the forms observed *in vivo*.[26,36] The cleavage site at Val-111 is located directly at the border of the IGFBP-like and Kazal-like domain (Figure 6) and is represented mostly at shorter incubation times. Further proteolysis produces the Gln-131 and Leu-150 N-termini, suggesting that initial proteolytic events are required for effective exposure to continued autolysis. The Leu-150 form (lower band) contains Cys-155 and thus differs from commonly produced ΔN-HtrA1

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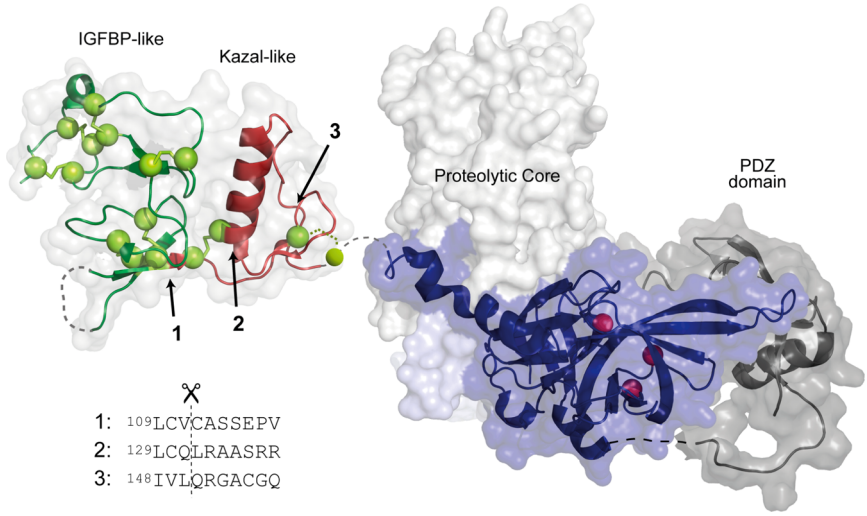

Figure 6. Structural representation of HtrA1 with the indicated locations of the three primary N-termini generated by autolysis. The HtrA1 domain architecture is visualized by representation of the structures for the N-domain [Protein Data Bank (PDB) entry 3TJQ[30]], the trimeric proteolytic core (PDB entry 3NWU[29]), and the PDZ domain (PDB entry 2YTW[48]). Disulfide bridges are shown as yellow spheres, and the catalytic triad is shown as red spheres. The cut sites resulting in the three major autolytic forms are numbered from 1 to 3, and their position in the folded N-domain is indicated with arrows. The new N-termini were identified by Edman degradation, and the surrounding sequence for each cut site is shown below the structure. Cut sites 1 and 2 are the result of cleavages with cysteine at position P1.

that lacks this residue. The loss of disulfide bridge integrity is likely to compromise the structural stability of the N-domain, possibly leading to partial unfolding. However, the sequential autolysis toward smaller autolytic forms suggests that the process is guided rather than being a random process facilitated by unfolding. Thus, our findings indicate that the N-domain is proteolytically processed to various degrees, correlated to the exposure time in an effective reductive environment that exposes suitable cysteine cleavage sites.

Intracellular HtrA1 is exposed to the ubiquitous cellular redox regulators TRX, GSH, and glutaredoxin. Of particular interest in this study is the finding that TRX potentiates N-domain degradation, supporting a correlation among intracellular HtrA1, redox balance, and autolysis. Furthermore, it suggests that the presence of HtrA1 autolytic forms is indicative of high intracellular (or extracellular) endogenous TRX levels. Most studies, in which HtrA1 autolysis has been observed, have been conducted in cancer cell lines, primarily to investigate the pro-apoptotic behavior of HtrA1 and the consequences of HtrA1 knock-down or forced expression.[8,26,36,37] It is important to note that increased intracellular levels of TRX and TRX reductase have been associated with several cancer cell types, providing an explanation for the observed autolysis of HtrA1 in such settings.[38−42] Treatment with the chemotherapeutic agents cisplatin and paclitaxel in ovarian cancer cell lines leads to upregulation of endogenous intracellular HtrA1 (and HtrA2), and this stimulates apoptosis through the targeted breakdown of XIAP.[37] Interestingly, a potential role for autolytic products of HtrA1 in the induction of apoptosis has been suggested because the expression of ΔN-HtrA1 in SKOV3 cells led to an increased level of apoptosis compared to that of WT HtrA1.[17,36] Recently, reduced levels of the autolytic ∼38 kDa product of HtrA1 were found to correlate with neoplastic bladder tissue undergoing cancerous transformation, which indeed highlights the importance of understanding the functional maturation of HtrA1 and the potential prognostic value of the autolytic forms.[28]

Several physiological and pathological conditions may result in increased amounts of local or systemic intra- and extracellular TRX, at levels that directly can impact HtrA1 autolysis. This is likely to be the case in human placentas complicated by preeclampsia where the presence of an autolytic 31 kDa form of HtrA1 correlates with the severity of the condition.[18] Preeclamptic placentas display alterations in the global redox balance, and the levels of antioxidant molecules (TRX and glutaredoxin) are elevated in response to increased oxidative insults,[43,44] thereby providing a possible explanation for the enhanced HtrA1 autolysis. In general, plasma levels of all HtrA family members are regulated during gestation,[45] and an autolytic form of HtrA3 (contains an N-domain similar to HtrA1) can be observed in plasma, suggesting that autolysis goes beyond the intracellular environment.[46,47] For HtrA1, it still remains to be established whether direct correlations exist between autolysis and intra- or extracellular location and how functional differences of the forms play a role.

On the basis of the findings in this study, we suggest that the observed autolysis of HtrA1 correlates with the redox balance of the system, in particular the levels of TRX. Our data show that HtrA1 specifically cleaves residues in its N-terminal domain after reduction of cysteine residues. Whether this cysteine cleavage property is used in other cellular settings is yet to be explored. Future studies should aim to resolve the functional aspect of the N-domain of HtrA1 but also how this secreted protein ends up in the intracellular compartment and whether such an event is linked to autolysis in any way.

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Funding

This work was supported by grants from the Danish National Research Foundation, the Danish Research Council for

Strategic Research/Natural Sciences, and the National Eye Institute (R01 EY012712).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DTT, dithiothreitol; GSH, reduced glutathione; HtrA1, high-temperature requirement A1; IAA, iodoacetamide; IGFBP, insulin-like growth factor binding protein; TBS, Tris-buffered saline; TRX, thioredoxin; WT, wild type; XIAP, X-linked inhibitor of apoptosis.

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