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HtrA3 is a cellular partner of cytoskeleton proteins and TCP1α chaperonin

Tomasz Wenta[a], Dorota Zurawa-Janicka[a], Michal Rychlowski[b], Miroslaw Jarzab[a], Przemyslaw Glaza[a], Andrea Lipinska[b], Krystyna Bienkowska-Szewczyk[b], Anna Herman-Antosiewicz[c], Joanna Skorko-Glonek[a], Barbara Lipinska[a,*]

[a] Department of General and Medical Biochemistry, Faculty of Biology, University of Gdansk, Poland
[b] Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology University of Gdansk - Medical University of Gdansk, Gdansk, Poland
[c] Department of Medical Biology and Genetics, Faculty of Biology, University of Gdansk, Poland

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ABSTRACT

The human HtrA3 protease is involved in placentation, mitochondrial homeostasis, stimulation of apoptosis and proposed to be a tumor suppressor. Molecular mechanisms of the HtrA3 functions are poorly understood and knowledge concerning its cellular targets is very limited. There are two HtrA3 isoforms, the long (HtrA3L) and short (HtrA3S). Upon stress, their N-terminal domains are removed, resulting in the more active ΔN-HtrA3. By pull down and mass spectrometry techniques, we identified a panel of putative ΔN-HtrA3L/S substrates. We confirmed that ΔN-HtrA3L/S formed complexes with actin, β-tubulin, vimentin and TCP1α *in vitro* and in a cell and partially co-localized with the actin and vimentin filaments, microtubules and TCP1α in a cell. *In vitro*, both isoforms cleaved the cytoskeleton proteins, promoted tubulin polymerization and displayed chaperone-like activity, with ΔN-HtrA3S being more efficient in proteolysis and ΔN-HtrA3L – in polymerization. TCP1α, essential for the actin and tubulin folding, was directly bound by the ΔN-HtrA3L/S but not cleaved. These results indicate that actin, β-tubulin, vimentin, and TCP1α are HtrA3 cellular partners and suggest that HtrA3 may influence cytoskeleton dynamics. They also suggest different roles of the HtrA3 isoforms and a possibility that HtrA3 protease may also function as a co-chaperone.

Significance: The HtrA3 protease stimulates apoptosis and is proposed to be a tumor suppressor and a therapeutic target, however little is known about its function at the molecular level and very few HtrA3 physiological substrates have been identified so far. Furthermore, HtrA3 is the only member of the HtrA family of proteins which, apart from the long isoform possessing the PD and PDZ domains (HtrA3L), has a short isoform (HtrA3S) lacking the PDZ domain. In this work we identified a large panel (about 150) of the tentative HtrA3L/S cellular partners which provides a good basis for further research concerning the HtrA3 function. We have shown that the cytoskeleton proteins actin, β-tubulin and vimentin, and the TCP1α chaperonin are cellular partners of both HtrA3 isoforms. Our findings indicate that HtrA3 may promote destabilization of the actin and vimentin cytoskeleton and suggest that it may influence the dynamics of the microtubule network, with the HtrA3S being more efficient in cytoskeleton protein cleavage and HtrA3L – in tubulin polymerization. Also, we have shown for the first time that HtrA3 has a chaperone-like, holdase activity *in vitro* - activity typical for co-chaperone proteins. The proposed HtrA3 influence on the cytoskeleton dynamics may be one of the ways in which HtrA3 promotes cell death and affects cancerogenesis. We believe that the results of this study provide a new insight into the role of HtrA3 in a cell and further confirm the notion that HtrA3 should be considered as a target of new anti-cancer therapies.

1. Introduction

Human HtrA3 is a member of the HtrA (High-temperature requirement A) family of serine proteases, which are well conserved in evolution. They degrade proteins with aberrant structure and also specific native proteins, and thus function as protein quality controllers

Abbreviations: CS, citrate synthase; ELISA, enzyme-linked immunosorbent assay; HtrA, High-temperature requirement A; MAPs, microtubule associated proteins; MST, MicroScale Thermophoresis; MT, microtubule; PD, protease domain; PDZ domain, postsynaptic density protein 95 *Drosophila* disc large tumor suppressor and Zonula occludens-1 protein domain; TCP1α, T-complex protein 1 subunit α

* Corresponding author at: Department of General and Medical Biochemistry, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland.
E-mail address: barbara.lipinska@biol.ug.edu.pl (B. Lipinska).

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and regulators of many cellular pathways (reviewed by [1–3]). Their common structural features are the presence of the chymotrypsin-like protease domain (PD) and at least one PDZ (Post-synaptic density 95, *Drosophila* discs large, Zonula occludens-1) domain localized at the C-terminus. The N-terminus is variable, may contain signal and regulatory sequences but is not required for proteolytic activity. PDZ domains may bind substrate proteins and/or regulatory peptides and thus act as substrate specificity determinants and regulatory elements [1,4–6].

Some of the HtrA proteins have been shown to possess, apart from the protease, a chaperone activity. The best-known example of such protease-chaperone is the HtrA (DegP) protein of *Escherichia coli* which may promote protein folding [6,7] or protect proteins from denaturation and aggregation by binding them (reviewed by [3,8–10]). The latter resembles holdase activity of the ATP-independent chaperones, e.g. sHsps, which may cooperate with the chaperones promoting *de novo* folding and refolding through ATP-regulated binding and release cycles, such as the HSP70s, HSP90s and the chaperonins (HSP60s) [11].

There are four human HtrAs, HtrA1-4, which play important functions in cellular physiology and are involved in several pathological processes (reviewed in [3,12,13]). HtrA3 is unique among the HtrAs since it has two isoforms: the long 49 kDa one (HtrA3L) and the short 36 kDa one (HtrA3S), produced by an alternative RNA splicing. The HtrA3 isoforms may exist in a cell separately or both variants may be present depending on tissue type [14–19]. So far a functional difference between the HtrA3L and HtrA3S forms remains an open question.

HtrA3L consists of a signal secretory peptide at the N-terminus, an N-terminal domain possessing a motif with homology to the insulin-like growth factor binding proteins (IGFBD) and a Kazal-type inhibitor motif (KI), followed by PD with the catalytic triad composed of His191, Asp227 and Ser305, and one C-terminal PDZ domain. In contrast, HtrA3S lacks the PDZ domain [14]. The IGFBD and KI motifs share homology with Mac25 protein and are termed a Mac25 domain. The HtrA3 N-terminal domain is not required for the protease activity and its removal causes activity increase [20]. Upon induction of apoptosis, the HtrA3 proteins undergo autocleavage of the N-terminal Mac25 domain [19–22]. The cleavage of the N-terminal domain from HtrA3L is necessary for mitochondrial to cytoplasmic translocation of the protease and increased cell death. Both the full length (residues 18–453) and N-terminally-truncated (residues ~130–453) forms of HtrA3L exist *in vivo* [19,22]. The PDZ domain is not required for proteolysis since in the *in vitro* experiments proteolytic activity of the HtrA3L and HtrA3S was similar [21,23], deletion of the PDZ domain did not impair the activity of HtrA3L, and the PDZ domain had no apparent effect on proteolysis at a wide range of temperatures [23].

HtrA3 was initially identified as a pregnancy-associated protease [14,24] and later on shown to be involved in embryo implantation and development of placenta [25–27], in TGFβ signaling and extracellular matrix modification [20], mitochondrial stability [28], apoptosis [19] and oncogenesis [19,29].

HtrA3 is localized extracellularly, due to the presence of the N-terminal signal peptide [14] and also intracellularly, in mitochondria and cytoplasm [19,30]. The mechanism governing the HtrA3 transport into the mitochondrium is not known, however Beleford et al. [19] suggested that it depends on the presence of the N-terminal Mac25 domain since the exogenous variant of HtrA3 lacking the Mac25 region has been found in the cytoplasm.

HtrA3 functions as a pro-apoptotic protein involved in the intrinsic, mitochondria-mediated apoptotic pathway. Upon action of apoptosis-inducing agents, it undergoes autoproteolysis resulting in the removal of the Mac25 domain and the processed HtrA3 (ΔN-HtrA3) is released into the cytosol where it promotes apoptotic cell death [19]. Very little is known about molecular events following the HtrA3 release to the cytoplasm and its cytoplasmic targets connecting HtrA3 to apoptosis have not been identified yet. So far it has been shown that Bcl-2 overexpression attenuates the HtrA3-mediated apoptosis [19].

Involvement of HtrA3 in apoptosis links this protein with cancer development. Dysfunction of HtrA3 was correlated with oncogenesis and it is suggested that the protease may act as a tumor suppressor. Downregulation of *HtrA3* was observed in several cancer cell lines and tumors such as ovarian, endometrial and lung cancers [15,16,18,29,31]. It has been demonstrated that HtrA3 proteolytic activity is indispensable to trigger lung cancer cell death caused by chemotherapeutic drugs [19] and proposed that silencing the HtrA3 gene could contribute to the etiology of chemoresistant disease in smoking-related lung cancer [29]. Furthermore, it has recently been shown that in lung cancer patients HtrA3 suppresses tumor cell invasiveness [32].

In spite of the indisputable fact that HtrA3 plays an important role in cell physiology and pathology, little is known about its function at the molecular level and very few HtrA3 physiological substrates have been identified so far. It has been shown in cell culture model that extracellular HtrA3 antagonizes HtrA4-mediated trophoblast invasion by degrading HtrA4 [27]. The recombinant HtrA3 was demonstrated to bind the purified growth factors of TGFβ family and cleave several extracellular matrix proteins [20]. The pull-down and immunoprecipitation experiments revealed that myosin-9 interacts with HtrA3 [21]. HtrA3 also degrades mitochondrial DNA polymerase gamma (POLG1) [28].

To gain insight into the molecular mechanism of the HtrA3 function in a cell, in this study we set out to identify cellular partners of the HtrA3L and HtrA3S isoforms. We concentrated on the N-terminally truncated, ΔN-HtrA3L/S proteins, since they have been shown to function in apoptosis [19], the process whose dysregulation is tightly connected to cancer development.

By the pull-down and mass spectrometry assays we were able to identify a large panel (about 150) of the HtrA3L/S potential partners. Out of these, for further studies, we chose a small group of proteins, encompassing the cytoskeleton proteins, such as actin, β-tubulin and vimentin, and the TCP1α chaperonin, which is a subunit of the CCT (TRiC) complex, indispensable for the actin and tubulin folding [33]. We confirmed that the cytoskeleton proteins and the TCP1α chaperonin are cellular partners of both HtrA3 isoforms. Moreover, *in vitro* the ΔN-HtrA3L/S stimulated tubulin polymerization and displayed chaperone-like activity. Based on these findings, we propose that HtrA3 influences cytoskeleton stability and thus may promote apoptosis. We also believe that the presented panel of the putative HtrA3 interacting proteins may serve as the basis for further research concerning HtrA3 cellular functions.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 ligase were purchased from Fermentas (Vilnius, Lithuania); primers used in site–directed mutagenesis were purchased from Genomed S.A. (Warszawa, Poland). Other chemicals, unless otherwise stated, were from Sigma-Aldrich (Poznan, Poland). The following antibodies were obtained from Sigma: mouse monoclonal anti-TCP1α, HRP-conjugated mouse monoclonal anti-β-actin, rabbit polyclonal anti-GFP, mouse monoclonal anti-vimentin and TRITC (tetramethylrhodamine)-conjugated phalloidin. Rabbit polyclonal antibodies against HtrA3 (PA1-41149), mouse monoclonal antibodies against β-tubulin and goat monoclonal antibodies against TCP1α were obtained from Thermo Fisher Scientific (Rockford, USA). Rabbit polyclonal anti-HtrA3 IgG (ab221369) and recombinant human β-tubulin protein were purchased from Abcam (Cambridge, UK), and recombinant human vimentin and actin proteins were from Cloud-Clone Corp. (Houston, USA).