Biochemical characterization of the HtrA2 and HtrA3 human proapoptotic proteases
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HtrA2 and HtrA3 proteins belong to the very well conserved in evolution HtrA (High temperature requirement A) family of serine proteases. Their characteristic features are the presence of protease domain (PD) and at least one PDZ domain at the C-terminus. In humans, four HtrA proteins (HtrA1-4) were identified of which HtrA3 exists as two isoforms, a long (HtrA3L) and a short one (HtrA3S), lacking the PDZ domain. The physiological significance of both isoforms is unknown. It has been shown that HtrA2 and HtrA3 proteases play an important role in cellular physiology. They are involved in the maintenance of mitochondrial homeostasis and stimulation of cell death by apoptosis. The dysfunction of their action are linked to severe diseases, including oncogenesis. It has been reported that the N-terminal domains of these proteases are not important for the proteolytic activity; during stress conditions they are removed which results in formation of the truncated ΔN-HtrA2 and ΔN-HtrA3 proteins.

According to the hypothetical model of the HtrA2 protease activation, the PDZ domains regulate the access to the catalytic center and during the activation process they change their position relative to PD. The aim of the first part of this work was to experimentally verify this model. To decrease the PDZ-PD interactions in HtrA2, a set of ΔN-HtrA2 variants was constructed, in which chosen amino acid residues located at the PDZ-PD interface were substituted for other, with opposite chemical properties. The influence of the substitutions on proteolytic activity was assayed with β-casein and fluorogenic peptide as substrates. It has been shown that the activity of the ΔN-HtrA2 V226K, ΔN-HtrA2 D302A, ΔN-HtrA2 R337L, ΔN-HtrA2 I373N, ΔN-HtrA2 E376L, ΔN-HtrA2 L377E, ΔN-HtrA2 R380L, ΔN-HtrA2 E425L and ΔN-HtrA2 R432L proteins was increased at a wide range of temperatures in comparison to the ΔN-HtrA2. These results together with kinetic assays suggest that, destabilization of both intra- and intersubunit interactions between PD and PDZ promote activation. These data support the theoretical HtrA2 activation model.

Next, biochemical characterization of, both HtrA3 isoforms, was performed. It was found that proteolytic activity of the ΔN-HtrA3S was similar to that of the ΔN-HtrA3L, which suggests that PDZ domain is not required for HtrA3 activity. Next, the influence of the unique interactions of the LB structural loop residues with PDZ domain (i.e. R362, E371, Q389) on
ΔN-HtrA3L activity, was investigated. The obtained results indicate that the LB residues and their interactions with PDZ domain promote activity with a peptide substrate.

As a part of the work aimed at finding HtrA3 activating peptides, it was shown that two peptides, X1 and X2, designed and synthesized by the Team of prof. A. Lesner (Faculty of Chemistry, University of Gdansk), efficiently activated the ΔN-HtrA3L protease. Approximately 50 % increase of activity was observed at 0,3 μM concentration of the X1 peptide.

Next, the putative cellular partners of both HtrA3 isoforms were identified, using “pull down” assay and mass spectrometry technique, and the lysates of the lung cancer A549 cells. It was found that the proteolytically inactive recombinant ΔN-HtrAL and ΔN-HtrAS could interact with the structural cytoskeleton proteins, proteins involved in maintenance of cellular homeostasis, ubiquitination, DNA repair, regulation of cell death, DNA replication, RNA modification, and intercellular transport connected with endocytosis. The further work was focused on verification of the cytoskeleton proteins, chaperone protein TCP1 (involved in cytoskeleton formation), and anti-apoptotic protein XIAP as the HtrA3 interacting partners. It has been shown, using specific antibodies, that the proteolytically inactive ΔN-HtrA3 isoforms formed complexes in vitro with the cellular actin, β-tubulin, vimentin, TCP1 and XIAP. Then, by using immunoprecipitation and western blotting techniques, it was confirmed that the endogenous HtrA3, from the HCC827 and SKBR3 cell lines interacted in vivo with these proteins. To check whether, both isoforms of ΔN-HtrA3 formed complexes with actin, β-tubulin, vimentin, TCP1 and XIAP in vivo, co-immunoprecipitation with exogenous proteolytically inactive ΔN-HtrA3L-GFP and ΔN-HtrA3S-GFP was performed. Western blotting assays confirmed interactions of the investigated proteins with the both ΔN-HtrA3 proteins.

Using confocal fluorescence microscopy it was shown that endogenous HtrA3 protein in the HCC827 and SKBR cells co-localized with actin, β-tubulin, vimentin, TCP1 and XIAP. Additionally, a similar assay was performed using the A549 cells, exogenously expressing ΔN-HtrA3S-GFP, confirmed co-localization of the investigated proteins with the short HtrA3 isoform.

Existence of direct interactions between the ΔN-HtrA3 isoforms and the tested proteins were confirmed by the enzyme-linked immunosorbent assay (ELISA). Moreover, using the MST (MicroScale Thermophoresis) assay, the efficiency of binding HtrA3 to XIAP
and TCP1 was analyzed. It was found that ΔN-HtrA3S interacted slightly more efficiently with XIAP than ΔN-HtrA3L. No difference in binding efficiency was observed for TCP1.

Furthermore, it was shown that both recombinant ΔN-HtrA3 isoforms cleaved β-tubulin, actin, vimentin and XIAP from the A549 cell lysates. TCP1 interacted with HtrA3 *in vivo* and *in vitro* but was not cleaved.

As an attempt to explain physiological significance of the HtrA3 interactions with the cytoskeleton proteins, the influence of both ΔN-HtrA3 isoforms on tubulin polymerization process was investigated. It was found that the proteolytically inactive and active ΔN-HtrA3 isoforms stimulated tubulin polymerization *in vitro*. It was confirmed, using transmission electron microscopy that ΔN-HtrA3L promoted formation of filamentary structures similar to microtubules.

The results presented above indicate that actin, β-tubulin, vimentin, TCP1 and XIAP are physiological, cellular partners of both HtrA3 isoforms and suggest that HtrA3 may be involved in modeling of cytoskeleton.

In conclusion, the obtained results provide a new insight into the function of the HtrA3 protease *in vitro* and *in vivo*. They could help to better understand the role of this protease in physiological and pathological conditions.