„Intracellular functions of the HtrA protein in the bacterium Helicobacter pylori'

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Maintaining protein homeostasis is crucial for the functioning of an organism. Bacteria have evolved sphisticated protein quality control (SKJB) systems that include chaperones, protein folding catalysts and proteases that degrade irreversibly damaged proteins. In Gram-negative bacteria, the main periplasmic protease is HtrA (high temperature requirement A protein). The functions of this protein have been best explored in *Enterobacteriaceae*, in particular in the model bacterium *Escherichia coli*. However, the role of the HtrA homolog in the cell of the bacterium *Helicobacter pylori* ($HtrA_{Hp}$) has not been the subject of detailed research so far, and the interests of researchers have focused primarily on the activity of the extracellular $HtrA_{Hp}$ as an important virulence factor of this human pathogen. The intracellular role of $HtrA_{Hp}$ in H. pylori seems to be particularly important, as so far the $htrA_{Hp}$ mutations have been introduced into only one strain of this species of bacteria.

The objective of the study was to determine the function of the $HtrA_{Hp}$ protein in the *H. pylori* cell. Specific objectives included the identification of proteins that are substrates and/or partners of $HtrA_{Hp}$ in the cell of *H. pylori* and the study of the impact of the $HtrA_{Hp}$ absence on the proteome of bacterial cells.

In the first stage of the research, using *in vivo* cross-linking of proteins combined with "pull down" and mass spectrometry techniques, the *H. pylori* proteins capable of binding to $HtrA_{Hp}$ were identified. Among them, a significant fraction were outer membrane proteins (OMPs), proteins involved in stress response and elements of the protein quality control system, proteins involved in the transport of metal ions, proteins responsible
for chemotaxis and mobility. In addition, proteins involved in a variety of cytoplasmic housekeeping processes were identified. The latter group appears to be non-specifically associated HtrAHp proteins.

In the next step, an attempt was made to verify the potential interactions of $HtrA_{Hp}$ with selected proteins. Putative elements of the periplasmic protein quality control system, folding catalyst HP_0175 (SurA-like protein), and proteases HP_0657 (YmxG), HP_1012 (PqqE) and HP_1350 were selected for the study. Immunoblotting of the elution fractions after the "pull down" experiment with anti-HP_0657, anti-HP_1012

and anti-HP_1350 antibodies partially confirmed the interactions between the tested proteins.

In order to verify the interactions of $HtrA_{Hp}$ with selected proteins, *in vitro* experiments were carried out using purified preparations of HP_0175, HP_0657, HP_1012, HP_1350 proteins. The results of the size exclusion chromatography did not allow to clearly demonstrate the presence of $HtrA_{Hp}$-tested protein complexes, while changes in $HtrA_{Hp}$ elution profiles suggested the presence of at least transient interactions.

Analysis of the level of HP_0657, HP_1012, HP_1350 proteins in the *H. pylori htrA* cells (*ΔhtrA*, *htrA S221A*) showed that the membrane-enriched fraction of the $HtrA_{Hp}$-deficient strain contained elevated levels of HP_0657 and HP_1012 proteins. Since none of these proteases were digested by $HtrA_{Hp}$ *in vitro*, it can be assumed that the accumulation of PqqE and YmxG is not due to the lack of degradation by $HtrA_{Hp}$, but rather to the need to replace the function of $HtrA_{Hp}$ and bind proteins with an abnormal structure.

Searching for substrates for the proteolytic activity of $HtrA_{Hp}$, it was found that $HtrA_{Hp}$ is capable of degrading an important *H. pylori* virulence factor, the CagA toxin. Another potential $HtrA_{Hp}$ protease substrate could be FrpB, a protein associated with the transport of iron ions, as well as other outer membrane proteins, Omp9 and Omp19, which also co-purified with $HtrA_{Hp}$ in the "pull down" experiments. However, the possibility of degradation of CagA and membrane proteins by $HtrA_{Hp}$ requires further verification.

To better understand the mechanisms involved in the $HtrA_{Hp}$ protein, quantitative SWATH-MS proteomics analyzes were performed. Changes in protein levels in lysates and membrane-enriched fractions obtained from a strain lacking the $htrA_{Hp}$ gene (*H. pylori* N6 *ΔhtrA*) against the proteome of the complementary strain (*H. pylori* N6 *ΔhtrA/htrA*) were examined. Among the proteins with a significantly changed content in the $\Delta htrA_{Hp}$ strain were OMPs, proteins involved in the stress response and elements of the protein quality control system, proteins involved in the transport of metal ions, proteins responsible for chemotaxis and mobility, proteins involved in in the metabolism and transport of amino acids, nucleotides, carbohydrates, lipids and basic metabolism. This result proves the pleiotropic effect of the $\Delta htrA_{Hp}$ mutation, which may be associated with disturbances in the biogenesis of the outer membrane. The

changed properties of the outer membrane in the *htrA*$_{Hp}$-deficient strain, suggesting a reduced integrity of this structure, were confirmed by the SDS ionic detergent sensitivity test - the *ΔhtrA*$_{Hp}$ cells showed greater susceptibility to lysis in the presence of detergent. In addition, *ΔhtrA*$_{Hp}$ bacteria were characterized by reduced surface hydrophobicity.

In conclusion, the results obtained in this study indicate that the functions performed by HtrA$_{Hp}$ in the *H. pylori* cell are very important for ensuring cellular homeostasis, necessary in the process of infection of the host organism. HtrA$_{Hp}$ most likely interacts with other components of the extracytoplasmic protein quality control system, including the proteases YmxG, PqqE, HP_1350 and PPIase HP_0175. The research carried out as part of this work indicates the important role of HtrA$_{Hp}$ in the biogenesis of the outer membrane, maintaining homeostasis of metal ions and ensuring the appropriate level of some virulence factors.