Ph.D. Thesis

The interactions and autoregulation of the iron regulatory peptide hormone hepcidin

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Pécs, 2011

Abbreviations

A1AT: alpha-1 antitrypsin, **ATCUN**: amino terminal Cu(II)-Ni(II)-binding motif, **BMP**: Bone morphogenetic protein, **BMPRE**: Bone morphogenetic protein response element, **C/EBPα**: CCAAT- enhancer binding protein α, **CD**: Circular Dichroism, **EPO**: erythropoietin, **EPOR**: erythropoetin receptor, **FP**: ferroportin, **GAPDH**: Glyceraldehyde 3-phosphate dehydrogenase, **GDF15**: erythroid factor growth differentiation factor 15, **GST**: glutathione S-transferase, **HAMP**: Hepcidin antimicrobial peptide, **HFE**: human hemochromatosis protein, **HIF1α**: Hypoxia inducible factor 1 α, **HJV**: hemojuvelin, HRE: Hypoxia response element, **IL-6**: Interleukin-6, **IRE**: Iron Resposive Element, **IRP**: Iron Regulatory Protein, **LB agar plate**: Luria-Bertani agar plate, **LEAP-1**: liver-expressed antimicrobial peptide, **MALDI TOF**: Matrix Assisted Laser Desorption Ionisation Time Of Flight, **NE**: nuclear extract, **NLS**: Nuclear localisation signal, **NMR**: Nuclear magnetic resonance, **PSSM**: Position-Specific Scoring Matrix, **sHJV**: soluble hemojuvelin, **SMAD7**: Mothers against decapentaplegic homolog 7, **STAT3**: Signal transducer and activator of transcription 3, **SVM**: Support Vector Machine, **Tf**: Transferrin, **TfR**: Transferrin receptor, **TGF-β**: Transforming Growth Factor-β, **TMPRSS6**: Transmembrane protease, serine 6, **TWSG1**: Twisted Gastrulation cytokine

Introduction

Hepcidin is the key iron regulatory hormone produced mainly in the liver and secreted into the blood. It was originally described as Liver Expressed Antimicrobial Protein (LEAP-1), an antimicrobial peptide exhibiting consistent antifungal but only weak antibacterial activity. Shortly after the discovery of hepcidin its fundamental role in iron homeostasis was realized. The hormone acts by binding to the iron exporter ferroportin, triggering its internalization and intracellular degradation. In the presence of hepcidin enterocytes release less iron into the portal system, resulting in downregulation of the iron uptake through the intestines. Similarly, hepcidin negatively regulates the macrophage iron export. Due to these effects, hepcidin overexpression has been convincingly linked to microcytic anemia.

Hepcidin is encoded by the *HAMP* gene and synthesized in hepatocytes as preprohepcidin. It is synthesized as an 84 amino acid (AA) preprohormone, and is present in the plasma not only as a mature 25 AA peptide, but in a 60 AA prohormone form as well. The maturation is facilitated by the serine peptidase furin. The aim of the present report is to reveal that prepro- and prohormones have significant interactions with proteins, which may

affect the maturation of the hormone in the cell and its cleavage to active hormone in blood. Iron is one of the essential trace elements in living organisms. In vertebrates plasma iron level is in the micromolar range and circulating iron is associated predominantly with transport protein transferrin. The blood iron levels, as well as the saturation of transferrin are frequently used indicators of the body iron status. Both iron deficiency and iron overload are potentially dangerous conditions, which may cause either anemia, enzyme dysfunctions, or degenerative liver, spleen and kidney diseases. The most important organs and tissues involved in the regulation of iron stores are the liver, placenta, intestine, and the macrophages. Recent findings indicate, that the hormone hepcidin plays a major part in controlling iron homeostasis. This peptide is synthesized in the liver as an 84 AA preprohormone, and is targeted to the secretion pathway by a 24 AA N-terminal targeting sequence. The resulting 60 AA prohepcidin is processed further into mature C-terminal 25 AA active peptide. The maturation is facilitated by serine protease furin. Furin belongs to the prohormone convertase family which recognizes the consensus sequence of R(X/R/K)(X/R/K)R.

Hepcidin regulates blood iron level through its interaction with ferroportin, an iron exporter molecule. Ferroportin is expressed in hepatocytes, duodenal enterocytes, and macrophages. After binding hepcidin, ferroportin is internalized, phosphorylated, ubiquitinated, and degraded by the target cells. As a result, hepcidin increases cellular iron sequestration, and reduces the iron level in the plasma. Hepcidin-independent regulation is also functionally important in vertebrates. Ferroportin expression was shown to be regulated translationally by intracellular iron through the IRE/IRP system and transcriptionally by heme, and these responses allow macrophages to match their iron export capacity to the fluctuating iron and heme load caused by episodic erythrophagocytosis. There are two major isoforms of ferroportin: FPN 1A, with a 5’IRE for translational repression in iron-deficient cells, and FPN 1B, lacking this motif. FPN 1B is expressed in duodenal enterocytes where it may allow them to export iron to the rest of the organism even if the enterocytes become iron-deficient.

The known signals for the induction of hepcidin synthesis are the elevation of plasma iron level, inflammations, and bacterial invasions. Consequently hepcidin increases iron sequestration and lowers the iron level in the blood. A structure-function study suggests that the N-terminus of hepcidin is essential for its interaction with ferroportin.

Mass spectrometry and chemical analysis revealed that the hepcidin peptide contains 8 conserved cysteines which are all involved in disulfide bonds. Three-dimensional nuclear magnetic resonance (3D-NMR) studies of the 20 and 25 amino acid form of hepcidin show

that their structure is a simple hairpin with eight cysteines that form four disulfide bonds in a ladder-like configuration, including an unusual disulfide bond that connects two adjacent cysteines (vicinal disulfide bridge). The peptide has an antiparallel β-sheet with a markedly amphipathic structure. The disulfide bond connectivity is a key determinant of the correct folding of the peptide and may have a critical role in hepcidin function.

The NMR structure of hepcidin-25 at different temperatures and its disulfide connectivity has been recently revised. Structural studies have shown that removal of individual disulfide bonds by pairwise substitution of cysteines with alanines, is quite permissive to changes, because the peptides exhibited almost full activity *in vitro*. Moreover, removal of disulfide bonds one at a time did not cause major changes in the peptide structure when analyzed by circular dichroism spectroscopy.

The N-terminal region of hepcidin-25 represents a metal binding site specific for the coordination of Cu(II) and Ni(II) known as ATCUN motif, and recently the great affinity of the N-terminal region of Hepcidin-25 for the copper has been confirmed. Although it is still not known the biological role of a metal binding motif in the hepcidin-25, its presence may be physiologically relevant, because has been recently demonstrated that the first five amino acids, starting from the N-terminus (DTHFP) are essential but not sufficient for its interaction with ferroportin, since their serial deletion, up to hepcidin-20, resulted in a gradual loss of activity.

Under physiological conditions the expression of the *HAMP* gene in the liver is modulated by numerous factors. The known positive regulators are the hereditary hemochromatosis protein (HFE), transferrin receptor 2 (TfR2), hemojuvelin (HJV) and bone morphogenetic proteins (BMPs). In addition, hepcidin expression can be regulated by factors independent of body iron levels, such as erythroid factors, hypoxia and inflammation. Relatively little is known about the suppression mechanisms of hepcidin synthesis. One of the identified negative regulators of liver hepcidin expression is matriptase-2 encoded by the TMPRSS6 gene. Matriptase-2 is a transmembrane serine protease, which inhibits the activation of hepcidin expression by interacting with membrane HJV and cleaving it into fragments. Recently SMAD7 was described as a potent inhibitor of *HAMP* gene expression. SMAD7 is an inhibitory SMAD protein that mediates a negative feedback loop to both TGF-β and BMP signaling.

Although both the regulation of the *HAMP* gene at transcriptional level and the role of mature hepcidin peptide have been studied extensively, little is known about the fate of precursor prohepcidin within the hepatocytes and in the blood. Prohepcidin was first

described as a nuclear peptide with a predicted nuclear localization signal (NLS) sequence, but later it was found mainly in the Golgi compartment, or in the cytoplasm showing granular cytoplasmic localization.

In general, mutations in regulatory molecules cause either hepcidin deficiency resulting in iron overload, or hepcidin excess with consequent iron deficiency and sequestration. HFE, transferrin receptor 2 (TfR2) and hemojuvelin (HJV) are genes mutated in human hemochromatosis, and their ablation results in decreased hepcidin responsiveness to iron, and relative or absolute hepcidin deficiency. Conversely, mutations in the protease TMPRSS6, which are associated with severe iron deficiency, prevent the appropriate decrease of hepcidin in the face of iron deficiency. Ferroportin mutations most often cause autosomal dominant “ferroportin disease”,manifested as loss of ferroportin function, iron accumulation in macrophages with very high levels of serum ferritin, but mild if any liver injury. Rare ferroportin mutations cause gain of function due to resistance to hepcidin, with parenchymal iron overload similar to that seen in classical hereditary hemochromatosis.

Hepcidin deficiency has been described in hereditary hemochromatosis (HH) caused by mutations in the hepcidin gene (HAMP). Five mutations affecting mature hepcidin have been identified to date. These mutations are G71D, C70R, C78T, C82Y and K83R (refer to the prepropeptide numbering system). The G71D amino acid substitution is a possible modifier in patients with hemochromatosis carrying HFE C282Y mutations. The K83R mutation in hepcidin was found in a patient suffering from porphyria cutanea tarda and iron overload (unpublished observation). Synthetic G71D and K83R peptides were fully functional *in vitro* assays. Substitutions affecting C70, C78 and C82 cysteines that form disulfide bonds in the mature hepcidin cause hemochromatosis. The C70R and C78T mutations which affect the third disulfide bond in hepcidin were described in patients with severe juvenile hemochromatosis. The C82Y heterozygous mutation with heterozygous R59D mutation cause adult onset hemochromatosis.

Objectives

To date, the only proven interaction of hepcidin is with the iron exporter molecule ferroportin. First we examined whether the disulfide bonds in hepcidin peptide play a role in ferroportin-hepcidin interaction. Next, we focused on identifying new protein–protein interactions of preprohepcidin, prohepcidin and hepcidin *in vivo*.

The main purposes of the work are:

- To create four hepcidin mutants by changing one of the cysteine coding nucleotide triplets (TGC) to serine coding triplet (TCC).
- To examine the interactions between mutant hepcidin peptides and ferroportin.
- To investigate the effect of mutant hepcidin peptides on ferroportin internalization.
- To identify new protein-protein interactions of preprohepcidin, prohepcidin and hepcidin using the BacterioMatch Two-Hybrid System.
- To confirm the protein-protein interactions by *in vitro* and *in vivo* methods.
- To examine these associations on hepatocyte cell line.
- To analyze the confirmed interactions in human serum.

Later on, we investigated the regulation of hepcidin expression. Although both the transcriptional regulation of the *HAMP* gene and the role of mature hepcidin peptide have been studied extensively, little is known about the fate of precursor prohepcidin within the hepatocytes and in the blood. Prohepcidin was first described as a nuclear peptide with a predicted nuclear localization signal (NLS) sequence, but later it was found mainly in the Golgi compartment, or in the cytoplasm showing granular cytoplasmic localization. Further aims of the work are:

- To describe the intracellular localization of prohepcidin.
- To determine the DNA binding motif of prohepcidin.
- To investigate the role of prohepcidin in the nucleus.
- To confirm that prohepcidin binds to its promoter *HAMP*.
- To examine the effect of prohepcidin overexpression and silencing on the activity of *HAMP* promoter.
- To explore the effect of A1AT on hepcidin expression.

Results

The role of hepcidin structure in the hepcidin-ferroportin interaction and in hepcidin-mediated ferroportin internalization

Mature hepcidin contains eight cysteine residues at the positions 7, 10, 11, 13, 14, 19, 22 and 23 which forms four disulfide bridges in a ladder-like configuration. The disulfide bonds evolve between the C7-C23, C10-C22, C11-C19 cysteine residues and between the

adjacent cysteines C13-C14 forming a vicinal disulfide bridge. These disulfide bonds are important in the formation of the stable hairpin structure with a distorted beta sheet, exhibiting an overall amphipathic structure.

To investigate the importance of disulfide bridges in the biological role of hepcidin, we created four mutant hepcidin by systematically mutating one cystein (TGC) to serine (TCC) in each peptide (sequence). The mutated hepcidin peptides were labeled as M1, M2, M3 and M4 in order to the position of the amino acid substitution. In the first mutant (M1) the C7 cysteine of the first disulfide bridge was mutated to serine, in the second mutant (M2) the C10 amino acid was substituted to serine. The M3 and M4 mutants harbor a mutation in the C11 amino acid or in the C13 amino acid crucial in the formation of the third or the fourth disulfide bridge, respectively.

We analyzed the amino acid sequence of the four mutant hepcidin peptides by I-TASSER server. The prediction shows that the amino acid change (C7 to S) in the first disulfide bridge redistributed the overall original molecular structure. The C10-C22 bridge remained intact, but the other disulfide bonds rearranged to C13-C19 bridge and for that reason, the vicinal bridge was missing. Unlike all the other mutant hepcidin peptides and the wild type hepcidin, M1 did not form β-sheet and the N and C termini diverged. Change of the C10 cysteine to serine eliminated the second disulfide bridge in the peptide and the vicinal bridge was not able to evolve. The lack of C11 resulted in a new S-S bridge realigned between C13 and C19 while the absence of C13 did not influence the first three disulfide bonds formation.

We examined the *in vivo* ability of the mutated hepcidin peptides (M1-M4) to bind to the whole ferroportin molecule using BacterioMatch Two-Hybrid system. The wild type hepcidin and ferroportin expressing bacterial cells gave strong growth rate, indicating that the mature hepcidin interacts with the whole ferroportin, as expected. We observed similar growth rates when the M2, M3 or M4 mutant hepcidin was expressed in the bacteria instead of the wild type hepcidin, while the bacterial cells co-transformed with ferroportin and M1 mutant hepcidin did not growth at all. This observation suggests that the disulfide bridge between the 7 and the 23 cysteine residues of hepcidin has a pivotal role in the interaction between hepcidin and ferroportin.

To obtain an independent support for the crucial importance of the first disulfide bridge in the interaction between hepcidin and ferroportin, we examined if the mutant hepcidin peptides expressed in hepatocytes are able to bind to ferroportin located in the hepatocyte cell membrane. WRL68 cells were transfected with preprohepcidin or with one of

the mutant preprohepcidin sequence containing pTriex3-Neo plasmid DNA. After 24 hours we collected the medium and determined its hepcidin concentration to prove that the posttranslational maturation of preprohepcidin occurred and the mature peptides were secreted. These expressed hepcidin-containing media were added to untransfected WRL68 cells. After 6 hrs treatment, the concentration of hepcidin in the media were determined again to evaluate the ability of secreted hepcidin to bind to ferroportin located in the cell membrane of untransfected WRL68 cells. We compared the level of expressed hepcidin protein in the media after 6 h and 24 h transfection. The significantly increased level of hepcidin after 24 h clearly decreased. The amount of hepcidin in the media collected from wild type or mutated hepcidin-overexpressing cells elevated compared to the hepcidin level of the medium of untransfected cells proving increased hepcidin expression and secretion due to transfection (129-222% compared to the control cells). When we transferred these media onto nontransfected WRL68 cells for 6 h, the levels of secreted wild type, M2, M3 and M4 hepcidin decreased to 82%, 82%, 74% and 47% respectively. This change was due to the fact that the peptide bound to ferroportin. On the contrary, there was no significant change observed in the secreted hepcidin peptide concentration in case of M1 hepcidin peptide. These data corroborate with our previous results indicating that the M1 mutant lacking the first disulfide bridge was not able to bind to its receptor ferroportin.

Since the secreted hepcidin regulates iron homeostasis by interacting with ferroportin, which results in internalization of ferroportin and loss of its function, finally we tested the ability of the mutant hepcidin peptides to induce ferroportin internalization *in vivo*.

WRL68 cells were transfected with preprohepcidin or with one of the mutant preprohepcidin coding pTriex3-Neo plasmid and were treated the same way as described above. Cells were fractionated after 6 h and 24 h long treatment with secreted hepcidin peptides containing medium. Then the isolated cytosol and membrane fractions were probed with anti-FP antibody. Untreated WRL cells were used as negative control and cells treated with wild type hepcidin containing medium were used as positive control for the internalization assay. Internalization of ferroportin was generated by mature hepcidin within 6 h. As it was expected, the M1 hepcidin internalization was failed, since this mutant was not able to bind to ferroportin. Surprisingly the other three mutant peptides M2, M3, M4 were not able to generate the internalization of ferroportin either.

To confirm the result of the internalization assay the iron (FeII) content of the treated WRL cells was determined. The results corroborated our previous observation: only the WRL

cells treated with wild type hepcidin showed elevated iron concentration compared to the control cells. Treatment with the mutant hepcidin peptides had no effect on iron export.

Summarizing our results, here we demonstrated that all the four intact disulfide bridges in hepcidin peptide are essential to its internalization activity, and the first disulfide bond has a crucial role in hepcidin-ferroportin interaction.

Interactions of preprohepcidin and prohepcidin with alpha 1-antitrypsin

The interactions of preprohepcidin and hepcidin with hepatocyte proteins were screening with BacterioMatch Two-Hybrid system. Preprohepcidin exhibited binding to transthyretin (or prealbumin), a serum protein known as a thyroid hormone carrier molecule. We also found the association of preprohepcidin with a-1 acid protein (orosomucoid), a major plasma protein with unknown function. The level of this protein is elevated in the blood in the case of inflammation, and it is used as a diagnostic marker in inflammatory diseases (acute phase protein). The strongest association of preprohepcidin proved to be with A1AT, a member of the serine protease inhibitor (serpin) family. A1AT was 'fished out' at the screenings more times than any other interacting protein (one-third of all sequenced cDNA clones), indicating a consistent and potentially relevant interaction with preprohepcidin. However, a more abundant representation of A1AT clones, when compared with other positive clones, cannot be excluded. The strong binding between preprohepcidin and A1AT was confirmed when the two proteins were co-expressed in BacterioMatch competent cells. As furin, a serine protease involved in the maturation of hepcidin, is also inhibited by A1AT, we considered this as a potentially important observation. This cotransformation was repeated with the same protease inhibitor and either the 60-AA prohepcidin (without the targeting sequence) or the 25-AA-containing mature hepcidin. We detected the growth of the cotransformed BacterioMatch strain in the case of prohepcidin, but not with mature hepcidin. We found that the protease inhibitor molecule binds selectively to the preprohormone and prohormone, but not to the processed hepcidin or to the targeting sequence of preprohepcidin.

There were other proteins (cytochrome P450, ATP⁄ADP translocase, enoyl-CoA hydratase) which gave weak interactions with preprohepcidin. Alignment of the coding regions of these proteins did not show significant similarities. Nor could we identify common structural domains that may provide further clues to preprohepcidin binding.

BacterioMatch screening carried out with the mature 25-AA peptide resulted in significantly fewer positive clones when compared with the screening with the

preprohormone. None of these proteins was identical with the screening results of the 84-AA peptide. The only strong and consistent interaction of the mature peptide was with membrane protein CD74. Further experiments are needed to evaluate this finding.

The most consistent and strongest interaction occurred with the serine protease inhibitor A1AT. This association was further tested by both *in vivo* and *in vitro* methods to evaluate its significance.

Both preprohepcidin and A1AT were cloned into inducible plasmids and expressed in bacteria. Preprohepcidin carried a glutathione S-transferase (GST) fusion tag for attachment to an affinity purification column. This column was used to pull down expressed A1AT from bacterial lysate or human serum. The interaction of A1AT with preprohepcidin was verified by the elution of protein complexes from the column, followed by western blotting developed with anti-A1AT IgG. The *in vitro* binding of the two molecules appeared to be specific, as GST-carrying affinity columns produced only negligible quantities of A1AT tethering.

Next, we studied the influence of the overexpression or downregulation of preprohepcidin on the A1AT mRNA level. We transfected WRL68 cells with preprohepcidin／pTriex3-Neo plasmid and were able to demonstrate a 470-fold increase in the copy number of preprohepcidin mRNA by real-time quantitative PCR. Using antisense RNA, we reduced the preprohepcidin mRNA level to 63%. The same samples were processed for A1AT mRNA level measurement. We found that the A1AT mRNA level increased by more than two-fold when preprohepcidin was overexpressed. Even more significantly, the 37% decrease in preprohepcidin expression caused by antisense RNA coincided with a nearly fourfold reduction of A1AT mRNA. These data suggest a regulatory link between the preprohormone and antiprotease expression, underlining a physiologically important relationship between the hormone and A1AT.

Next, we studied the interaction of prohepcidin and plasma A1AT in the circulation. We carried out ultrafiltration assays with sera collected from presumably healthy volunteers. After measuring the prohepcidin level with ELISA, the serum was filtered through a 30 kDa cut-off membrane and the prohepcidin level was determined in the filtrate (first ultrafiltrate). Prohepcidin itself did not bind to the filter of the Microcon tube, and A1AT did not appear in the serum ultrafiltrate (data not shown). We found that the serum prohepcidin level was 210 μg/ml, whereas the first filtrate contained 71.7 μg/ml (34% of the total). Although these data prove that normally more than 60% of the total prohepcidin is bound to serum proteins larger than 30 kDa, no evidence could be found that A1AT binds prohepcidin significantly. To demonstrate the capability for binding ‘free’ (filterable) prohepcidin to A1AT, the above

experiment was repeated after the addition of 1.5 g/L A1AT to the first serum ultrafiltrate. The prohepcidin concentration in the second ultrafiltrate was further reduced to 46.6 µg/ml (22% of the total), or to 65% of the first filtrate.

To reveal the specificity of the preceding binding reaction, we performed coimmunoprecipitation assays. We attached A1AT antibody to a column of CNBr activated Sepharose beads, and incubated this with serum. Sepharose beads were washed and A1AT-associated proteins were eluted with Laemmli buffer. Next, we probed the eluent with anti-hepcidin IgG. Results of the dot blot displayed strong positive signals, indicating that A1AT and prohepcidin associated *in vivo* in the serum. Ultrafiltrated 'free' prohepcidin by itself gave no binding to the activated Sepharose beads.

Similar affinity purification was carried out using the ZipTip method, in which A1AT antibody was attached to the C18 column of ZipTip and incubated with serum, as in the previous experiment. The eluted sample was analyzed on a MALDI-TOF mass spectrometer. The spectrum was compared with that obtained in the case of bacterially expressed His-tagged prohepcidin with a molecular weight of 7760.08 Da. In the latter case, two major peaks appeared in the spectrum, at 1410.96 and 6349.12 *m/z.* The peak at 1410.96 *m/z* corresponds to a fragment of 6X His and 5 AA from the C-terminal end of prohepcidin (MCCKTHHHHHH). The affinity-purified prohepcidin from serum gave the same 6349.14 *m/z* peak as above, suggesting a similar fragmentation of the prohormone. In this experiment, the C-terminal 5-AA (MCCKT) fragment does not appear, as detection was performed between 1000 *m/z* and 7500 *m/z* to exclude matrix peaks in the low mass ranges. Not only does this affinity purification assay reveal that A1AT binds prohepcidin, but it also confirms that the whole prohepcidin molecule is involved in the reaction.

Autoregulation: a new way of regulating prohepcidin expression

We transiently transfected WRL68 cells with pTriex3-Neo plasmid containing the *HAMP* gene in order to overexpress prohepcidin. Intracellular localization of prohepcidin was studied by using *in vivo* immunocytochemistry. The cells were labeled with anti-prohepcidin antibody and imaged using a laser scanning confocal microscope to allow imaging of cellular cross-sections. In addition to the granular cytoplasmic staining which may suggest that the peptide is concentrated within vesicles in the cytoplasm, prohepcidin was also found in the nuclear region of hepatocytes.

The nuclear localization of prohepcidin raised the possibility that it may act as a factor influencing its own gene expression. In order to predict the DNA binding properties of prohepcidin we used an SVM-based prediction algorithm. The results of the prediction for prohepcidin and hepcidin are shown in Table 1. The SVM scores for prohepcidin based on amino acid composition (1.47) and based on position-specific scoring matrix (PSSM) (1.07) were both higher than the threshold (1.0), corresponding to the highest specificity setting, predicting DNA binding by prohepcidin. Notably, the analysis of the 25-amino acid hepcidin sequence predicted no DNA binding (SVM scores 0.03 and 0.16).

To confirm the ability of prohepcidin to bind to DNA, we performed chromatin-immunoprecipitation assay (ChIP). Formaldehyde-crosslinked prohepcidin-bound DNA fragments were immunoprecipitated from WRL68 cells overexpressing prohepcidin-His peptide using anti-His antibody. ChIP assay revealed significant amount of *HAMP* promoter bound to prohepcidin, raising the possibility of an interaction between prohepcidin and the promoter region of its own gene.

To obtain independent support for the DNA binding property of prohepcidin, we developed a PCR-based promoter binding assay and we further analyzed the interaction between the peptide and the promoter region of *HAMP* gene. The promoter binding assay was performed using WRL68 cells overexpressing prohepcidin-His peptide. The peptide was purified from the nuclear extract using anti-His antibody-coated CNBr Sepharose beads, then the washed beads were incubated with *HAMP* promoter. The peptide-DNA complex was eluted from the surface of the beads, and the eluate was used as a template in a PCR reaction with promoter specific primers for the detection of prohepcidin-bound *HAMP* promoter. As our results show, we detected promoter binding only in the sample in which prohepcidin-His was incubated with the *HAMP* promoter. We did not observe promoter binding in case of controls.

In a similar experiment we first pre-incubated the *HAMP* promoter with the nuclear extract of WRL68 cells overexpressing prohepcidin-His peptide, and then we isolated the HAMP-prohepcidin-His complex using anti-His CNBr Sepharose beads. Similarly to our previous result, we detected promoter binding only from the sample in which the prohepcidin-His peptide and the *HAMP* promoter were present. These results clearly suggest a specific interaction between prohepcidin and its own promoter.

However, it is possible that prohepcidin did not bind to the beads alone, but as part of a larger complex that might contain other DNA-binding protein(s). The presence of such protein(s) could also explain the positive result of the *HAMP* promoter binding assay. To find

out whether prohepcidin can bind to the promoter region of the *HAMP* gene by itself or as a member of a complex, we expressed prohepcidin-His in BL21 bacterial cells in order to prevent the potential formation of a complex with nuclear DNA-binding proteins. The immunoprecipitated peptide was subjected to the promoter binding assay using exactly the same method as in previous experiments. Results show that we could amplify the *HAMP* promoter region only from the sample which contained the bacterially expressed prohepcidin-His peptide, while DNA binding was not detected in the control samples. These results confirm that prohepcidin alone is able to bind to the *HAMP* promoter.

Having identified prohepcidin as a *HAMP* promoter binding peptide, we turned our attention to confirming whether this interaction has a *HAMP* gene regulatory effect. To determine the effect of prohepcidin binding on the *HAMP* gene expression we compared the *HAMP* promoter activity at different intracellular prohepcidin levels.

To increase the constitutive level of prohepcidin in WRL68 cells, we transiently transfected the cells with pcDNA3.1 expression plasmid containing preprohepcidin cDNA. In addition, the intracellular prohepcidin level was reduced by transfecting the cells with pcDNA3.1 plasmid containing preprohepcidin antisense DNA. To help the quantification of the *HAMP* promoter activity we cloned the 942-bp fragment of the *HAMP* promoter as a transcriptional fusion with firefly luciferase, resulting in the pGL3/HAMP promoter construct that was cotransfected with the pcDNA3.1/preprohepcidin or the antisense construct into WRL68 cells. Expression levels of preprohepcidin mRNA were measured by real-time PCR and the activation of the promoter was determined by Luciferase assay.

At normal prohepcidin level the *HAMP* promoter-luciferase reporter vector exhibited basal activity in WRL68 hepatocytes. In cells harboring the overexpression construct the preprohepcidin mRNA level was 435-fold higher and the activity of the HAMP promoter decreased to 52% of the original level. An even more significant effect was detected upon diminishing the amount of prohepcidin. When we decreased the amount of intracellular prohepcidin to 63% of the normal level, the activity of the HAMP promoter increased up to 5.2-fold compared to the original level.

These results are specific for the pro form of the hormone, as overexpressing mature hepcidin in WRL68 cells did not result in decreased *HAMP* promoter activity. To verify whether the pro region alone is sufficient to influence the activity of the *HAMP* promoter, we created a fusion protein containing the pro region of prohepcidin fused to a cytoplasmic enzyme, GAPDH. Overexpression of this protein in WRL68 cells did not change the activity of the *HAMP* promoter, indicating that the pro region alone is not sufficient for the DNA

binding activity. Finally, the elevated or decreased level of prohepcidin had no effect on the activity of the promoter region of transin gene, indicating that the interaction between prohepcidin and HAMP promoter is specific. These observations suggest that prohepcidin regulates its own gene expression by downregulating its promoter activity.

We have previously shown that prohepcidin specifically binds to A1AT in the cell and in the serum, indicating the role of A1AT in the posttranslational modification of prohepcidin. In order to find out if this interaction has any influence on the *HAMP* gene expression, we increased the amount of A1AT in WRL68 cells and analyzed its effect on *HAMP* promoter activity using Luciferase assay. When we overexpressed A1AT, the activity of the *HAMP* promoter increased up to 3.3-fold compared to the *HAMP* promoter activity in cells with normal A1AT level. The elevated promoter activity suggests that the A1AT-bound prohepcidin may not be able to downregulate *HAMP* gene expression.

To study the effect of A1AT on the binding of prohepcidin to the *HAMP* promoter, we overexpressed A1AT in WRL68 cells, purified the protein by immunoprecipitation and analyzed the sample with dot blot using anti-hepcidin antibodies. As expected, we could detect not only the isolated A1AT, but the A1AT-bound prohepcidin as well. Next the immunoprecipitated A1AT-prohepcidin complex was subjected to a *HAMP* promoter binding assay. Our observation was that neither prohepcidin-free A1AT (bacterially expressed A1AT) nor A1AT-bound prohepcidin (from WRL68 cells) were able to bind to the *HAMP* promoter.

Taken together, these results suggest that only the non-A1AT-bound prohepcidin binds to the promoter site, and as a consequence, downregulates *HAMP* gene expression.

Summary

Hepcidin plays a crucial role in iron homeostasis. To date, the only well known protein which has an interaction with hepcidin is the iron exporter ferroportin. The hepcidin-mediated ferroportin internalization is well described, although the role of disulfide bonds of hepcidin peptide is still unknown.

- We proved that the first (C7-C23) disulfide bond is essential in hepcidin-ferroportin interaction.
- We demonstrated that all the four disulfide bridges are necessary for hepcidin-mediated ferroportin internalization. The substitution of one of the conserved cysteine residues eliminated the biological activity of hepcidin.

Taken together, these results suggest that in case of the known hepcidin mutations affecting the conserved cysteine residues (C70R and C78T) hepcidin is able to bind to ferroportin but not able to cause its internalization.

We were interested in identifying new protein-protein interactions of preprohepcidin, prohepcidin and mature hepcidin. These new interactions could influence on iron homeostasis and hepcidin regulation.

- We demonstrated that the serine protease A1AT gives strong interaction with preprohepcidin and prohepcidin.
- We also demonstrated an interaction between preprohepcidin and alpha 1- acid glycoprotein, and preprohepcidin and transthyretin.
- We proved that the mRNA expression of A1AT shows parallel pattern with preprohepcidin mRNA overexpression and silencing.
- We confirmed the specificity of A1AT-preprohepcidin interaction in hepatocyte cell line with *in vivo* (crosslinking) and *in vitro* (GST pull down) methods.
- We demonstrated that A1AT-prohepcidin binding is specific in the serum (ELISA, coimmunoprecipitation, serum ultafiltration assay, MALDI TOF).
- We proved that mature hepcidin shows strong interactions with ferritin heavy chain and CD74 protein.

Our results demonstrated that the serine protease inhibitor A1AT binds preprohepcidin within the cell during maturation. Furthermore, A1AT binds prohepcidin in the plasma. This observation may explain the presence of prohormone in the circulation, as well as the post-translational regulation of the mature hormone level in the blood.

Although both the regulation of the *HAMP* gene at transcriptional level and the role of mature hepcidin peptide have been studied extensively, little is known about the fate of precursor prohepcidin within the hepatocytes and in the blood. We aimed to determine the localization of prohepcidin within the hepatocytes and reveal its function.

- We showed that prohepcidin is located in the cytosol as well as in the nucleus of WRL68 hepatocyte cells.
- We confirmed that prohepcidin possesses a DNA-binding motif using SVM based algorithm.

- We proved that prohepcidin binds specifically to its own promoter *HAMP* by chromatin immunoprecipitation and by a protein-DNA binding method developed by our research group.
- We demonstrated that prohepcidin is able to bind to its promoter by itself without any other DNA-binding proteins.
- We confirmed that prohepcidin is able to regulate its own gene expression.
- We proved that changes in the level of A1AT influence *HAMP* gene expression.
- We demonstrated that prohepcidin bound to A1AT is not able to interact with *HAMP* promoter; therefore prohepcidin is not capable to regulate its own gene expression.

These results suggest that prohepcidin is localized not only to the cytoplasm but also to the nucleus of hepatocytes. The nuclearly located peptide binds to the *HAMP* promoter, causing decreased promoter activity. Moreover, the known interaction between A1AT and prohepcidin inhibits this autoregulatory effect. We described a novel role of prohepcidin in the regulation of *HAMP* gene expression which may contribute to better understanding of the regulation of iron homeostasis.

Acknowledgement

I would like to thank Dr. Katalin Sipos the support and guidance I received.
I would like to thank Prof. Dr. Attila Miseta and Prof. Dr. Gábor L. Kovács for managing my work and giving support and useful advices.
I would like to thank the technical help I recieved from all my colleagues at the Institute of Laboratory Medicine, the Department of Forensic Medicine and the Department of Biochemistry and Medical Chemistry.
I would like also thank dr. Zsuzsanna Fekete for the never ending encouragement and advices.
I would like to thank Dr. Beáta Polgár for her help and advices.

Publications

Publications in the topic/A témában közölt publikációk

Articles/Cikkek

E. Pandur, J. Nagy, V. S. Poór, Á. Sarnyai, A. Huszár, A. Miseta, K. Sipos:
Alpha-1 antitrypsin binds preprohepcidin intracellularly and prohepcidin in the serum
FEBS Journal. 2009; 276: 2012-2021. **IF 3,042**

J. Nagy, L. Lakner, V. S. Poór, **E. Pandur**, Gy. Mózsik, A. Miseta, K. Sipos:
Serum prohepcidin levels in chronic inflammatory bowel diseases
Journal of Crohn's and Colitis 2010; 4: 649-653. **IF 1,729**

E. Pandur, K. Sipos, J. Nagy, V.S. Poór, L. Grama, A. Miseta and Zs. Fekete:
Prohepcidin binds to *HAMP* promoter and regulates its own gene expression in WRL68 cell line
Biochimie. 2011;

E. Pandur, J. Nagy, V.S. Poór, Zs. Fekete, A. M. Peti, A. Miseta, K. Sipos:
The higher structure of hepcidin is essential for binding to ferroportin
Peptides. 2011;

Published abstracts/Idézhető absztraktok

E. Pandur, J. Nagy, V. S. Poór, A. Miseta, K. Sipos:
In vivo interactions of preprohepcidin and mature hepcidin with ferroportin
33. FEBS Congress Athens, Greece, June 28 -July 3. 2008. FEBS Journal. 2008; 275 s1: 316. **IF 3,14**

V. S. Poór, **E. Pandur,** J. Nagy, K. Sipos, A. Miseta:
Protein-protein interaction screening with BacterioMatch system
33. FEBS Congress, Athens, Greece, June 28 -July 3. 2008. FEBS Journal. 2008; 275 s1: 424. **IF 3,14**

J. Nagy, **E. Pandur**, V. S. Poór, B. Debreceni, K. Sipos:
The function of human RNase L Inhibitor in translation
33. FEBS Congress Athens, Greece June 28 -July 3. 2008. FEBS Journal 2008; 275. s1: 127. **IF 3,14**

Oral presentations/Előadások

Sipos K.; **Pandur E.**; Nagy J.; Poór V. S.: A hepcidin, egy vasanyagcserét szabályozó hormon jellemzése
37. Membrántranszport Konferencia Sümeg máj. 22-25. 2007.

V.S. Poór, **E. Pandur**, J. Nagy, A. Miseta, K. Sipos: New protein-protein interactions of the human hepcidin
The 5th International Conference of Postgraduate Medical Students Hradec Králové Czech Republic nov. 27-29. 2008.

Miseta A.; **Pandur E.**; Nagy J.; Huszár A.; Sipos K.: Az alfa-1 antitripszin szerepe a hepcidin érésében
Erdélyi Múzeum Egyesület, Orvos és Gyógyszerésztudományi Szakosztály, XIX. Tudományos Ülésszak Marosvásárhely Románia ápr. 23-25. 2009.

Posters/Poszterek

Pandur E.; Nagy J.; Szabó A.; Montskó G.; Radnai B.; Sipos K : A hepcidin expressziójának intracelluláris szabályozása
V. Magyar sejtanalitikai konferencia Budapest máj. 4-6. 2006.

Pandur E.; Nagy J.; Szabó A.; Montskó G.; Peti M. A.; Sipos K.: Ferroportin egy hepcidin hormon által szabályozott vas transzporter- **Előadásra kiválasztott poszter**
36. Membrán-Transzport konferencia Sümeg máj. 23-26. 2006.

Pandur E.; Nagy J.; Montskó G.; Peti M. A.; Sipos K.: A hepcidin, egy vasanyagcserét szabályozó hormon vizsgálata
A Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése Pécs aug. 30- szept. 2. 2006.

Pandur E.; Nagy J.; Poór V. S.; Peti M. A.; Sipos K.: Hepcidin interakciója más fehérjékkel
37. Membrán-Transzport konferencia Sümeg máj. 22-25. 2007.

Poór V. S.; **Pandur E**.; Nagy J.; Peti M. A.; Sipos K.; Miseta A.: A hepcidin antimikrobiális hatásmechanizmusának vizsgálata
37. Membrán-Transzport konferencia Sümeg máj. 22-25. 2007.

Pandur E.; Nagy J.; Poór V. S.; Sipos K.: C/EBP alfa és SMAD4 transzkripiós faktorok szerepe a vasanyagcsere szabályozásában WRL68 sejttenyészetben
XVI. Nemzetközi Semmelweis Szimpózium és VI. Magyar Sejtanalitikai Konferencia Budapest nov. 15-17. 2007.

Poór V. S.; **Pandur E**.; Nagy J.; Sipos K.: A hepcidin és ferroportin expressziójának változása CaCo, WRL és HepG2 sejtekben
XVI. Nemzetközi Semmelweis Szimpózium és VI. Magyar Sejtanalitikai Konferencia Budapest nov. 15-17. 2007.

Nagy J.; Lakner L.; **Pandur E**.; Poór V. S.; Mózsik Gy.; Sipos K.; Miseta A.: Prohepcidinszint meghatározása gyulladásos bélbetegségekben
XVI. Nemzetközi Semmelweis Szimpózium és VI. Magyar Sejtanalitikai Konferencia Budapest nov. 15-17. 2007.

Pandur E.; Nagy J.; Poór V. S.; Miseta A.; Sipos K.: A hepcidin és a ferroportin kapcsolatának vizsgálata BacterioMatch Two-Hybrid rendszerrel
38. Membrán-Transzport konferencia Sümeg máj. 20-23. 2008.

Nagy J.; **Pandur E**.; Poór V. S.; Rab A.; Sipos K.; Miseta A.: C/EBP alfa és SMAD4 transzkripciós faktorok hatása a hepcidin expressziójára
38. Membrán-Transzport konferencia Sümeg máj. 20-23. 2008.

Poór V. S.; **Pandur E**.; Nagy J.; Rideg O.; Miseta A.; Sipos K.: Protein-protein kapcsolatok vizsgálata BacterioMatch rendszerrel
38. Membrán-Transzport konferencia Sümeg máj. 20-23. 2008.

Pandur E., Nagy J., Poór V. S., Sarnyai Á., Rapp J., Miseta A., Sipos K.:A hepcidin promóterének aktivitás-vizsgálata luciferáz rendszerrel
A VIII. Magyar Genetikai Kongresszus és a XV. Sejt- és Fejlődésbiológiai Napok Nyíregyháza ápr. 17-19. 2009.

Poór V. S., **Pandur E**., Nagy J., Sarnyai Á., Miseta A., Sipos K.: A hepcidin kapcsolata két akut fázis fehérjével

A VIII. Magyar Genetikai Kongresszus és a XV. Sejt- és Fejlődésbiológiai Napok Nyíregyháza ápr. 17-19. 2009.

Pandur E.; Nagy J.; Poór V. S.; Montskó G.; Sarnyai Á.; Miseta A.; Sipos K.: Az α1-antitripszin szerepe a hepcidin érésében
39. Membrán-Transzport konferencia Sümeg máj. 19-22. 2009.

Poór V. S, **Pandur E**, Nagy J, Rapp J, Sarnyai Á, Miseta A, Sipos K: Hepcidin mutációk: a térbeli szerkezet és a biológiai aktivitás összefüggése
39. Membrán-Transzport Konferencia Sümeg máj. 19-22. 2009.

Nagy J, **Pandur E**, Poór V. S, Sarnyai Á, Miseta A, Sipos K: A hemszintézis és a hepcidin transzkripció szabályozásának kapcsolata
39. Membrán-Transzport Konferencia Sümeg máj. 19-22. 2009.

Frank D, **Pandur E**, Poór V. S, Nagy T, Sarnyai Á, Miseta A: A hepcidin kimutatása a nagy nyálmirigyekből
39. Membrán-Transzport Konferencia Sümeg máj. 19-22. 2009.

Pandur E., Nagy J., Poór V.S., Rapp J., Miseta A., Sipos K., Fekete Zs.: A hepcidin gén (HAMP) expresszió szabályozásának új mechanizmusa hepatocitákban - **Előadásra kiválasztott poszter**
40. Membrán-Transzport Konferencia Sümeg máj. 18-21. 2010.

Poór V.S., **Pandur E.,** Nagy J., Rácz E., Miseta A., Sipos K.: A hepcidin és az alfa-1 savas glikoprotein interakciója
40. Membrán-Transzport Konferencia Sümeg máj. 18-21. 2010.

Further posters/További poszterek

B. Sümegi; A. Pálfi; K. Kovács; K. Hantó; P. Deres; **E. Pandur**; E. Bognár; A. Toth; K. Toth: Akt/GSK-3β, PKC and MAP kinase cascades contribute to the protective effect of PARP inhibition
PARP 2005 Symposium Newcastle England oct. 5-7. 2005.

Pandur E.; Nagy J.; Szabó A.; Montskó G.; Sipos K.: A mitokondrium jelentősége a transzláció szabályozásában humán sejtekben
36. Membrán-Transzport konferencia Sümeg máj. 23-26. 2006.

Nagy J.; **Pandur E**.; Debreceni B.; Montskó G.; Sipos K.: Egy közös molekula szerepe a riboszómális RNS érésben élesztő és emlős sejtekben
A Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése Pécs aug. 30- szept. 2. 2006.

Nagy J.; **Pandur E**.; Debreceni B.; Poór V. S.; Sipos K.: Humán- élesztő hibrid RNáz inhibitor funkcionális vizsgálata
37. Membrán-Transzport konferencia Sümeg máj. 22-25. 2007.

Polgár B.; **Pandur E**.; Gajdos G.; Fekete Zs.; Berki T.; Jerry N. van Dijken P.; Szekeres-Barthó J.: PIBF specifikus poli- és monoklonális antitestek alkalmazása az immunbiológiai kutatásokban
Magyar Immunológiai Társaság XXXIX. Vándorgyűlése Szeged nov. 3-5. 2010.

Bogdán Á.; Polgár B.; **Pandur E.;** Szekeres-Barthó J.: A PIBF mRNS expressziós mintázatának vizsgálata terhességhez asszociált szövetekben
A Magyar Immunológiai Társaság XXXIX. Vándorgyűlése Szeged nov. 3-5. 2010.