Ph. D. Thesis

The examination of iron metabolism in chronic inflammatory bowel disease and the functional analysis of RNase L Inhibitor

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Abbreviations

ABC ATP binding cassette, **ATF6** activating transcription factor 6, **CD** Crohn's disease, **c/EBPa** CCAAT/enhancer-binding protein alpha, **CREBH** cyclic AMP response element-binding protein H, **DCYTB** Duodenal cytochrome B, **DMT1/Nramp2** divalent metal transporter 1/ natural resistance-associated macrophage protein 2, **DTT** Dithiothreitol, **ELISA** enzyme-linked immunosorbent assay, **EMEM** Eagle's minimal essential medium, **EOR** endoplasmic reticulum overload response, **ER** endoplasmic reticulum, **FBS** foetal bovin serum, **HAMP** hepcidin antimicrobial peptide, **IL-6** interleukin-6, **IRE** iron responsive element, **IRP** iron regulatory protein, **LPS** lipopolysacharid, **MMD** yeast minimal medium, **NBD** nucleotide-binding domain, **NOD2/CARD15** nucleotide-binding oligomerization domain containing 2/caspase recruitment 15, **PMSF** phenylmethanesulfonylfluoride, **Rli** RNase L inhibitor, **SDS-PAGE** sodium dodecyl sulfate polyacrylamide gel electrophoresis, **SERCA** sarco/endoplasmic reticulum Ca2+-ATPase, **STAT3** signal transducer and activator of transcription 3, **TfR** transferrin receptor, **UC** ulcerative colitis, **UPR** unfolded protein response, **UPRE** unfolded protein response element, **XBP** X box binding factor 1, **YNB** yeast nitrogen base, **YPD** Yeast Peptone Dextrose (media for growing yeast).

Introduction

Iron is one of the essential elements in all organisms. In mammals the majority of iron is incorporated into heme and iron-sulfur clusters, which molecules besides other functions are essential in respiration. The blood iron level in mammals is regulated by the peptide hormone hepcidin which is synthesized predominantly in the liver as an 84 amino acid preprohepcidin encoded by *HAMP* gene. Following cleavage and maturation the 25 amino acid active hormone is secreted into the blood. The main action of hepcidin is its binding to ferroportin, the only known iron exporter molecule, causing the internalization and degradation of ferroportin. The consequences of this mechanism will be the reduction of iron export from enterocytes, hepatocytes, and macrophages which process will lead to blood iron level decrease. The regulation of hepatic expression of hepcidin is controlled by the body iron status, anemia, hypoxia and inflammations.

Hepcidin is synthesized in the liver as a preprohormone, secreted as a 60 amino acid (AA) prohormone, and processed by furin to a 25 AA mature hormone. The details of this cleavage are not known yet, one possible regulator is alpha-1 antitrypsin which can bind to the prohormone and inhibits the maturation process. The mature hepcidin binds to its receptor ferroportin, an iron exporter transmembrane protein, which is found in the intestine, placenta and macrophages. The increased level of hepcidin blocks the iron absorption in the intestine and the iron release from the macrophages through the internalization of ferroportin, thus lowering the serum iron content. During inflammation body iron homeostasis is damaged and anemia will develop as a consequence of the illness. One of the major mediators of

inflammatory response is IL6 cytokine which is produced by macrophages and T cells. This cytokine induces the expression of the majority of acute phase proteins including hepcidin. This transcriptional regulation eventuates through the classical JAK-STAT pathway.

Crohn's disease (CD) and ulcerative colitis (UC) are the two most frequent chronic inflammatory bowel diseases (IBDs). They affect mainly children and middle-aged adults in the well-developed countries. In Crohn's disease any part of the gastrointestinal tract may be involved but most often the small bowel and sometimes the colon, therefore the presence of impaired absorption may occur. In ulcerative colitis the inflammation is limited to the large bowel. In the two diseases the histological findings are the following: in colitis ulcerosa only the mucosa and the submucosa are affected, while in Crohn's disease transmural inflammation is observed. Despite some fundamental differences in the characteristics of the two conditions, they have a few features in common. Clinical manifestations could be similar: abdominal pain, diarrhea, changes in certain laboratory parameters. The etiology of these disorders is complex. There are theories assuming that the development of IBD is the result of an inappropriate immune response to microorganisms.

In both conditions hypochromic anemia and low serum iron level will arise with time. The reasons of these complications are frequent intestinal bleedings, malabsorption, and the high level of inflammatory cytokines which contribute to the so called anemia of chronic disease or anemia of inflammation.

The development of anemia of chronic disease (ACD) is the result of numerous factors. One of them is hepcidin, a peptide hormone which is the main regulator of iron homeostasis. Hepcidin mRNA expression is regulated by the iron status of the body, hypoxia, and inflammation. Besides playing an important part in iron metabolism, hepcidin also has a direct antimicrobial effect.

Lately it has been proved that other pathways for hepcidin induction exist which act through the activation of unfolded protein response (UPR). ER stress induced transcription factors CHOP, C/EBPα and CREBH are described to be involved in *HAMP* gene transcription activation. The activation of UPR in mammals is generated through the action of ER stress sensors IRE1, ATF6 and PERK. They all have influence on downstream signaling molecules. IRE1 is built up of four distinct domains. The N-terminal ER lumenal domain is proposed to sense the accumulation of unfolded proteins. Beside a transmembrane domain IRE1 contains a cytoplasmic kinase and an endonuclease domain. The kinase domain is responsible for the autophosphorylation of Ire1p which will lead to the activation of the endonuclease domain. The substrate of the endonuclease domain is the mRNA of Xbp which will be spliced as the result of the enzymatic activity of Ire1p. The spliced form of Xbp is an active transcription factor which is able to bind to the promoters of ER chaperones and ERAD components.

Within the cells the major site of iron assembling into molecules is the mitochondria. Iron-sulfur clusters for extramitochondrial proteins are build in the mitochondria as well. So far the only essential cytosolic iron-sulfur protein, which is not involved in the iron-sulfur cluster biosynthesis is the RNase L inhibitor, Rli. First Rli was recognized as the inhibitor of RNase L, an 2-5A activated nuclease, which degrades single-stranded viral and cellular RNAs. Later it became obvious that Rli has functions in almost every step in translation: ribosomal RNA

maturation and export, cytosolic ribosome biogenesis, translation termination and ribosome recycling.

It is noteworthy to mention that RNase L has a kinase-like and an RNase domains which show sequence similarities to the kinase and nuclease domains of Ire1p. It is tempting to suppose that Rli may have inhibitory effect on the activity of Ire1p. In this paper we present a novel function of human Rli, the involvement in the activation of UPR. We prove that after the reduction of Rli expression in HeLa cells, the amount of spliced *XBP* mRNA significantly increases in the cells. Also it was demonstrated by us that low level of Rli1p in HeLa cells gave rise to increased *HAMP* gene expression. This latter observation may indicate a connection between iron-sulfur cluster biosynthesis and hepcidin expression regulation.

Aims

1. Our study aimed at unraveling whether the serum prohepcidin level can provide a useful diagnostic parameter in Crohn's disease and colitis ulcerosa.
2. Structural analysis of human and yeast Rli: the differences and their consequences.
3. Examination of already known yeast functions of Rli in human cells in protein synthesis, ribosome assembly and unfolded protein response.
4. Relationship between the expression of hepcidin and the activity of unfolded protein response.

Results

1. Measure of serum prohormone concentrations in two inflammatory bowel diseases

114 patients participated in our clinical and laboratory studies including 12 healthy volunteers (control group). Of the 114 patients 72 (42 women and 30 men) were diagnosed with ulcerative colitis, while 30 patients (15 women and 15 men) suffered from Crohn's disease. The mean age of the study group was 41.375 years (range 19-84 years). Serum samples were collected and clinical parameters were obtained from each participant. All the patients and the volunteers were informed about the study and gave written informed consent in accordance with the regulations of the local Ethics Committee. Crohn's disease and ulcerative colitis activity indexes were calculated according to published methods. Serum prohormone concentrations were determined using an enzyme-linked immunosorbent assay, Hepcidin Prohormone ELISA kit (DRG International, Inc., USA) according to the manufacturer's protocol.

Of the serum factors indicating the presence of inflammation C-reactive protein level and the erythrocyte sedimentation rate were significantly higher ($P<0.01$) in both disease groups than in the healthy people. Among the parameters revealing the iron supply of the patients only serum iron level ($P<0,001$) and transferrin saturation ($P<0.01$), a derivative of this value were significantly lower in people suffering from CD or UC compared to healthy controls.

61.5% and 44.4% of the patients with CD and UC, respectively, had serum iron level below 14μmol/L, the others presented the normal values. We performed statistical analyses of these subgroups. However, this division did not change the correlations discussed above.

The other laboratory parameters determined (leukocyte count, serum albumin, total iron-binding capacity, hemoglobin, and transferrin) were in the normal range in most cases, but showed relatively high standard deviations in both groups. Prohepcidin levels measured by competitive ELISA did not show statistical differences between CD and UC patients or healthy and ill persons. Elevated prohepcidin levels (>240 ng/mL) were measured in the 19.38% of the patients, while 9.8% had reduced prohepcidin levels (<80 ng/mL).

Endoscopic control examinations were not carried out routinely. The calculated activity indexes were based on clinical parameters. Regarding the activity indexes few patients were in active status of the diseases (AI>150), only 19.38% of the two groups (4 patients with CD, 15 patients with UC). The majority of the patients (80.6%) were in remission.

Serum transferrin levels and transferrin saturation significantly correlated with prohepcidin levels in CD (P=0.04) and showed weaker correlation in UC (P=0.05). The total iron binding capacity showed low correlation with prohepcidin levels in CD patients (P=0.05). There was no significant correlation between the levels of prohepcidin and serum iron or hemoglobin contents in neither of the diseases.

Although C-reactive protein levels and erythrocyte sedimentation rates were significantly higher in UC than in the healthy controls, these parameters did not show any correlation with prohepcidin measurements (P>0.05). The activity indexes, leukocyte count and albumin levels were not correlated with serum prohepcidin level, either (P>0.05).

In the case of Crohn's disease, there was a positive but weak correlation between prohepcidin levels and the activity index. Serum albumin also correlated weakly with prohepcidin levels. Serum transaminase activities were found in the normal range in the majority of the cases (data not shown).

2. Study with RNase L Inhibitor

2.1. Comparison of human and yeast Rli protein sequences

As human Rli was not able to complement its yeast homologue, we focused on and analysed the sequence similarities of these proteins. The protein sequence alignment was performed by ClustalW software and the result was analysed with GeneDoc program.
The statistic report indicated 67% homology/identity and 82% similarity and revealed 18% difference between the two Rli proteins. The difference in protein length was 9 AA (1%).
We found two larger regions closer to the N terminus showing high divergence, in addition to a moderately divergent region closer to the C terminus, while the rest of the sequences seemed to be highly conserved.

2.2. Human Rli cannot complement Tet-Rli in budding yeast cells

Previous studies have revealed that the deletion of Rli1p gene in Saccharomyces cerevisiae is lethal. Therefore, to be able to investigate the lack of Rli1p, we have previously constructed a yeast strain (Tet-Rli) in which Rli mRNA level was controlled under a tetracycline-repressible promoter. On rich medium, in the presence of tetracycline derivative doxycycline, Tet-Rli cells exhibit growth arrest because of the decreased Rli1p level. As yeast and human Rli proteins share high level of homology it was tempting to try whether the human protein will compensate for the reduced level of Rli1p in yeast cells. In the complementation assay Tet-Rli yeast cells were transformed with yeast Rli1/pRS424 or human Rli/pRS424 expression vectors then treated with doxycycline. The Tet-Rli yeast cells exhibited strong growth after transformation with yeast Rli/pRS424 in the presence of doxycycline. By contrast, the cells expressing the human Rli protein did not grow in the presence of doxycycline, indicating, that the expression of human Rli protein could not compensate for the lack of the original yeast Rli1p.

2.3. The role of highly divergent regions in the function of Rli1p

Rli proteins are composed of four main domains, two ABC-type nucleotide binding domains (NBD) and two cysteine-rich motifs at the N-termini which bind two iron-sulfur clusters. To find out which region is responsible for the functional differences between the human and the yeast homologue, we created six chimera proteins, in which the fusion regions were not at the domain limits but according to the highly conserved sequences. Therefore, the N termini of chimera 1, 3 and 5 contain the yeast sequences until AA 173, AA 221 and AA 372, and these sequences were fused with the 435, 387 and 236 AA long C termini of human Rli sequence, respectively. Chimera 2, 4, and 6 consist of the human Rli sequences at their N termini, so their sequences started with the first 173, 221 and 372 AA of the human Rli sequence, which were fused with the 435, 387 and 236 AA long C termini of the yeast Rli1p, respectively.

These newly constructed chimeras were used in the complementation experiment/assay. We cloned all the six chimera sequences into pRS424 yeast expression vector and transformed them into Tet-Rli cells, then these strains were grown on Doxycycline containing rich medium.

Our results indicate that the first 173 AA region is not responsible for the functional differences at all, as replacing this part in the yeast protein with the human counterpart sequence did not have fatal consequences (ch 4). Contrary, when we replaced the N terminal part of yeast protein containing the first divergent region with its human counterpart (ch 5), or when we replaced the yeast C terminal 236 AA (including a divergent region) with its human counterpart (ch 3), the result of the complementation experiment showed, that these regions were indispensable for the yeast protein, because none of the above chimeras could grow in the presence of doxycycline. However, these regions alone were not sufficient for the yeast cells, as neither chimera 2 containing the first divergent yeast region, nor chimera 6 containing the third yeast region could successfully replace the original Rli1p. In case of the

second divergent region our results suggest that this region is not enough for the complementation even in combination with the first or with the third region (ch 3 and ch 5).

These data clearly declare, that despite their overall high homology, Rli1p contains more highly divergent regions indispensable for the function of the yeast protein. All these divergent regions are crucial, as replacing them with the human counterpart sequence failed yeast growing in each cases. This raises the possibility that not just one specific region, but rather the the higher molecular structure of Rli proteins are responsible for the functional differences.

2.4. Effects of Rli chimera proteins on ribosomal RNA content of Tet-Rli1 cells

Next we examined the effects of chimera Rli proteins expression on ribosomal RNA levels in Doxycycline treated Tet-Rli1 cells. Yeast cells were transformed with expression vectors as before and total RNA were isolated from the Tet-Rli1 cells. RNA was loaded onto agarose gel and 18 S and 28 S rRNAs were visualized by ethidium bromide staining. Similarly to the previous experiment, only two strains had normal rRNA content: one which was transformed with yeast Rli1-pRS424, the other with that chimera (ch4) expressing vector, the only which was able to complement Rli depletion in Tet-Rli1 cells. We obtained similar results when we measured the ribosomal RNA levels of the strains using real time PCR. Similarly to the result of the complementation assay, only two strains had normal rRNA content: the strain in which the original yeast Rli1p was overexpressed and in which that chimera Rli was overexpressed, which was able to compensate for the loss of the original Rli1p (ch4).We obtained similar results when we measured the ribosomal RNA levels of the strains using real time PCR.

2.5. Downregulation of Rli in HeLa cells

For the examination of the consequences of reduced level of Rli in mammalian cells, first we compared the two most wildly used gene-silencing methods in mammalian cell lines: the antisense technique and the siRNA method. HeLa cells were transfected with an antisense Rli plasmid construct or with purified diced siRNA, cells were collected after 12 h or 24 h transfection, and Rli mRNA level was determined by real time PCR. Rli mRNA levels were diminished by both transfection methods, especially after 24 h. Even after 12 h transfection the Rli mRNA levels were reduced, and 24 h treatment gave significant Rli protein level reduction compared with the untreated cells suggesting that both methods are equaly suitable for the Rli level reduction in HeLa cells. Northern blot of HeLa cells transfected with antisense Rli (RAS) and Rli dicer for 24 h. Beta actin mRNA was used as loading control. To find the most powerful method to reduce the level of Rli in HeLa cells, we compared the endogen Rli protein level of three different human cell lines: HepG2, a hepatoma cell derivative, WRL68, an embryonic hepatic cell line, and HeLa cancer cell line. We performed Western blot analysis using anti-human Rli antiserum (data not shown). After loading equal amounts of total proteins onto SDS gels, the extract of HeLa cells gave the strongest reaction with anti-human Rli antiserum, suggesting that the expression level of Rli protein is the highest in HeLa cells among these three cell lines.

2.6. Protein synthesis is diminished in HeLa cells after decreased Rli expression

The yeast Rli1p has been demonstrated to perform in protein translation and ribosome biogenesis. The human homologue, ABCE1 was just recently proved to interact with eukaryotic initiation factors. To obtain further evidences, that the human Rli may be of importance for translation, we decreased Rli protein level of HeLa cells. After 24 h transfection with either antisense Rli/pCDNA 3.1 plasmid or purified diced siRNA HeLa cells were incubated with Isolabel ^{35}S-Methionine. Protein concentrations of cell lysates were determined and equal amounts of protein samples were loaded onto SDS gel. This reduction of translational efficiency is a general effect, and strengthens the fact that the depletion of Rli protein impairs protein synthesis in human cultured cells.

2.7. Effect of Rli depletion on the expression of ribosomal components in HeLa cells

Even though, the role of yeast Rli1p in ribosome biogenesis has been previously demonstrated, the role of the human homologue ABCE1 in the same process is still unknown. Since the two proteins share high homology and the human RLI was proved to be needed for translation, it is tempting to examine the possible role of this protein in the maturation of ribosomes. Hence, we analysed the consequences of human Rli protein depletion onto ribosomal RNA and protein content of HeLa cells. After Rli depletion total RNA was extracted, and ribosomal RNA levels were determined by Northern blot analyses and by real time PCR. The result of the Northern blot illustrates that Rli protein depletion caused reduced 5.8 S and 18 S rRNA expressions, and we obtained similar results with real time PCR concerning rRNA levels. It is intriguing that the mRNA levels of both ribosomal subunit proteins (Rpl 30 and Rps 3) increased following Rli protein depletion. These data suggest a role for human Rli in ribosome biogenesis similar to its yeast homologue.

2.8. Activation of XBP mRNA splicing, UPR in HeLa cells transfected with antisense-Rli

As yeast and human Rli do not complement each we searched for functional differences between the two proteins. Interestingly RNase L and IRE1p, which is one of the stress sensors of UPR, share a homology on their C-terminal part: the kinase and endonuclease domains of IRE1 are very similar to the kinase-like and nuclease domains of RNase L. We were curious whether because of this structural similarity human Rli1p may take part in the process of unfolded protein response (UPR).

To induce UPR, HeLa cells were treated with thapsigargin or with DTT. Thapsigargin treatment reduces ER Ca^{2+}-content, while DTT disrupts disulfide bonds in proteins triggering UPR. To follow UPR activation we determined total and spliced XBP mRNA levels by real time PCR. As it is seen on Fig 7 thapsigargin treatment caused 7-fold increase in XBP mRNA level, while the spliced XBP mRNA level rose 80-fold compared to the untreated cells. Upon DTT treatment we gained (obtained) the same effect on the levels of total and spliced XBP mRNA (of HeLa cells).

When HeLa cells were transfected with Rli antisense pCDNA3.1 and XBP (total and spliced) mRNA levels were measured with real time PCR, we observed the elevation of both types of mRNA the same way as in case of thapsigargin treatment. When thapsigargin was added together with Rli antisense transfection, the two effects. Taken together, these data led us to conclude that diminished Rli expression activates UPR in mammalian cells.

3. Activation of UPR had influence on hepcidin expression in HeLa cells

Lately there are evidences in the literature that the expression of hepcidin, the iron regulatory hormone of mammals is under the control of ER stress. It seems that the different effects which lead to UPR activation may influence hepcidin transcription through different intracellular signaling pathways. We wondered whether UPR activation via Rli depletion can cause the induction of hepcidin synthesis. To verify this assumption, we isolated total RNA from Rli antisense transfected or thapsigargin treated HeLa cells and analysed the expression of HAMP mRNA by real time PCR. Interestingly, thapsigargin treatment of HeLa cells by itself led to no change (did not change the level of hepcidin mRNA) in hepcidin mRNA level, while Rli depletion resulted in more than 4-fold increase in HAMP mRNA level. From these results we concluded that hepcidin expression is not increased by every type of UPR upregulation, but Rli depletion is one of the factors which can effect the HAMP gene transcription.

Discussion

1. Serum prohepcidin measurement in two inflammatory bowel diseases

The present study was undertaken to clarify whether prohepcidin ELISA measurement can provide some useful information on IBD. Our plan was to reveal any difference in the prohepcidin levels in colitis ulcerosa and Crohn's disease. Another aim of this study was to find relationship between prohepcidin serum levels and the development of anemia or presence of inflammation in chronic bowel diseases.

Patients with IBD demonstrate laboratory features of acute phase reaction and histological signs of leukocyte migration of the intestine. Normally the mucosal immune system generates immune response against pathogenic material while maintaining tolerance to self-antigens. Part of the unspecific antimicrobial system of mucosal cells in mammals is the peptide family of defensins. Hepcidin and defensins possess similar synthetic pathways and higher structures, like hairpin-shape molecules with 3-4 intramolecular disulfid bridges. In IBD levels of certain defensins are diminished, which raised the possibility that defensins may be involved in their pathogenesis. Though hepcidin has a definite antimicrobial activity, in this case its iron metabolism regulator effect is determinant.

Hepcidin is the major hormone regulator of iron homeostasis. This peptide is synthesized in the liver as an 84 amino acid preprohormone, and targeted at the secretion

pathway by a 24 amino acid N-terminal targeting sequence. The resulting 60 amino acid prohepcidin is processed further into mature C-terminal 25 amino acid active peptide.

The present study was also meant to find out whether the serum prohepcidin level could be used as a diagnostic or prognostic factor of Crohn's disease and colitis ulcerosa. We sought for any correlations between serum prohepcidin levels and laboratory parameters of iron homeostasis (serum iron, total iron binding capacity, hemoglobin, transferrin, and transferrin saturation), inflammatory parameters (C-reactive protein, leukocyte count, and erythrocyte sedimentation rate) or activity indexes.

A large number of previous studies deal with chronic disorders accompanied by iron metabolism disregulation especially by apparent anemia. A number of authors have tried to find relationship between prohepcidin or hepcidin serum levels and various laboratory parameters in hepatic cirrhosis, chronic renal failure, malignant diseases, rheumatoid arthritis, and systemic lupus erythematosus (SLE). In general, serum prohepcidin levels and hepcidin mRNA expression are decreased in hepatic illnesses (hepatocellular carcinoma, alcohol induced cirrhosis, chronic hepatitis C). In chronic renal failure the levels of prohepcidin and mature hepcidin are elevated. In the case of tumours other than hepatocellular carcinoma hepcidin expression is up-regulated, leading to anemia of chronic disease.

Recent studies indicate that in two chronic inflammatory diseases, rheumatoid arthritis (RA) and systemic lupus erythematosus the prohepcidin levels do not correlate with the disease activity scores, hemoglobin, serum iron and cytokine levels. In patients suffering from RA with high levels of ferritin correlations between serum prohepcidin level and IL-6 or rheumatoid factor titer were found.

In IBD patients included in the present study the C-reactive protein, erythrocyte sedimentation rate are significantly higher, while serum iron levels and transferrin saturation are significantly lower than the same parameters of the control (healthy) group. We demonstrated correlation between serum prohepcidin level and transferrin, transferrin saturation, total iron binding capacity, activity index and albumin in CD, and transferrin, transferrin saturation in UC. We did not carry out serum ferritin level determinations routinely, as in this study a relatively large number of patients were involved for several years. The relationship between prohepcidin and serum ferritin is already known, we were interested in uncovering new correlations.

However, it seems that the prohepcidin measurement itself does not provide useful information on the CD or UC status. It is known that the regulation of hepcidin expression is complex, the most important factors which determine serum hepcidin levels are the intracellular iron content, serum iron level, and inflammatory factors. While these stimuli have unequal relative weight, the effect of one can overrule that of another. The situation is more complex if we consider that hepcidin expression control is different in hepatic and erythropoietic cells. It is also recognized that serum hepcidin level may change rapidly, and hepatic hepcidin expression modifications are not always followed by peptide level shift. Further investigations are necessary to measure hepcidin in the blood and/or in the urine. These data are currently gathering and with time the conclusion will be clear.

2. Experiments with RNase L Inhibitor

In our study we presented a novel function of human RNase L inhibitor in the unfolded protein response of mammalian cultured cells. We also demonstrated that UPR activated by diminished Rli1p expression in HeLa cells has an effect in HAMP gene expression. This gene is encoded by hepcidin, the major iron metabolism regulator hormone in mammals.

We compared Rli mRNA levels in three different laboratory cell cultures, two of which originated from carcinomas. It is worth to mention that Rli protein expression was definitely higher in tumorous cell cultures than in the WRL 68 cell line. This raises the possibility that in cells with high proliferation activity and/or malignant transformation the Rli1p level is elevated. For the explanation of this phenomenon further experiments are needed.

The essential function of Rli1p in different steps of translation was first proved in yeast, but now it is obvious that Rli is indispensable in the protein synthesis of mammalian cells. In this report we demonstrated that the depressed Rli protein level concluded in reduced methionine incorporation into newly synthetized proteins in HeLa cells. This was a general effect, the synthesis of every types of proteins became defected in Rli-depleted HeLa cells. It seems intriguing that besides the above mentioned functional similarities of yeast and human Rli proteins, the latter one is not able to complement the Rli1p depressed yeast strain, though the two proteins possess high degree of structural relationship. It is feasible that the higher structure of human Rli protein differs from its yeast relative, which can cause functional differences.

During seeking for another potential interaction partner for Rli1p we became interested in IRE1. This protein is one of the mammalian ER stress sensor molecules located in the ER membrane. The cytoplasmic domain of IRE1 contains a kinase and an endonuclease domain, which feature both structural and functional similarities to those of RNase L. As human RLI is a known binding partner of RNase L, it is conceivable that Rli may have interaction with IRE as well.

In our experiments of Rli deprived HeLa cells we observed seven-fold increase of XBP mRNA and eighty-fold elevation of spliced XBP mRNA level compared to the untreated cells. Randal et al demonstrated that dimerized IRE1 caused *XBP* mRNA splicing in the cytoplasm without UPR activation. Similarly, by reducing Rli in HeLa cells, the RNase activity of IRE1 may be activated.

Just like in 2'-5' A pathway, where Rli binds to and inhibit the endonuclease activity of Rnase L, Rli may bind to IRE1, as well. This interaction may protect the dimerization of IRE1, thus may regulate its endonuclease activity. However, there is no direct proof of direct binding between these proteins, it is ceasable, that Rli may work as a potent inhibitor of IRE1 protein. It is not clear yet what is the exact relationship between Ire1 and Rli proteins. We are not aware of any direct connections between Ire and Rli molecules but this binding may exist in the cells as the endonuclease domain of Ire1p is located in the cytoplasm. By diminishing the amount of Rli, the RNase domain of Ire may become „unprotected". The outcome of such an event can be the unleashing of RNase activity of Ire1p.

Lately two independent studies revealed a connection between UPR activation and HAMP gene expression. In Science 2009 the authors demonstrated hepcidin expression induction following different treatments by ER stressors. The expression of mRNA from HAMP gene increased as the result of each chemical addition. Another group administered DTT to HepG2 cells and demonstrated hepcidin expression elevation. Others demonstrated increased hepcidin expression after DTT treatment. In our work we proved that the essential iron-sulphur protein Rli deprivation not only activated UPR pathway in HeLa cells, as shown in increased level of spliced XBP mRNA, but hepcidin gene expression was also induced. We do not know the exact explanation of this phenomenon at the moment, but we have preliminary experimental data which suggest a link between mitochondrial iron supply and hepcidin expression. The correct explanation of this phenomenon is not known so far, although we have preliminary data suggesting a link between hepcidin expression and mitochondrial iron supply. It seems possible that the Rli protein reduction may cause mitochondrial iron homeostasis disturbance which can lead to elevated hepcidin expression. Further studies are needed to elucidate this regulatory mechanism.

Summary

1. Of the serum factors indicating the presence of inflammation C-reactive protein level and the erythrocyte sedimentation rate were significantly higher in both disease groups than in the healthy control group. The serum iron level and transferrin saturation were significantly lower in people suffering from CD or UC compared to healthy controls.
2. Serum transferrin levels and transferrin saturation significantly correlated with prohepcidin levels in CD and showed weaker correlation in UC. The total iron binding capacity showed low correlation with prohepcidin levels in CD patients. In the case of Crohn's disease, there was a positive but weak correlation between prohepcidin levels and the activity index.
3. The expression of RNase L inhibitor is elevated in tumor cell lines compared to embryonic cell lines.
4. Although it the difference between the yeast and human Rli proteins is only 18%, the expression of human Rli protein could not compensate the lack of the original Rli1p in yeast.
5. The depletion of Rli protein impairs protein synthesis in human cultured cells; the reduction of translational efficiency is a general effect.
6. The results led us to conclude that diminished Rli expression activates UPR in mammalian cells.
7. The hepcidin expression is not increased by every type of UPR upregulation, but Rli depletion is one of the factors which can affect the HAMP gene transcription.

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Publikációs jegyzék/ Publications

A disszertáció alapját alkotó közlemények/ Publications in topic

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 2,628

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Témához kapcsolódó egyéb tudományos közlemények/ Other publications

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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. 2012

Referált folyóiratban megjelent absztraktok/ Citable abstracts

J. Nagy ; E. Pandur; V. S. Poór; B. Debreceni; K. Sipos: The function of human RNase L Inhibitor in translation.
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