

**VILNIUS UNIVERSITY  
INSTITUTE OF BIOTECHNOLOGY**

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**INTERACTION OF HEPATITIS B VIRUS CORE PROTEIN AND  
ITS MUTANT FORMS WITH HUMAN LIVER PROTEINS**

Summary of doctoral dissertation

Physical science, biochemistry (04 P), nucleic acids, protein biosynthesis (P 320)

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**VILNIAUS UNIVERSITETAS  
BIOTECHNOLOGIJOS INSTITUTAS**

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**HEPATITO B VIRUSO ŠERDIES BALTYMO IR JO MUTANTINIŲ  
FORMŲ SAŪVEIKA SU ŽMOGAUS KEPENŲ BALTYMAIS**

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## INTRODUCTION

Hepatitis B virus (HBV) remains a major health problem, causing various clinical manifestations from asymptomatic to fulminant and acute hepatitis. Chronic infection can develop to cirrhosis or hepatocellular carcinoma [3]. It is generally regarded that wild-type (wt) HBV is not directly cytopathic to liver cells, and liver disease is mediated mainly by host immune response [9]. However, it is known that HBV-infected long-term immunosuppressed patients may develop cirrhosis and end-stage liver disease, although in these cases immune-mediated mechanisms are unlikely to be significant. It is already shown that the progression of liver disease in long-term immunosuppressed kidney transplant recipients is associated with accumulation of hepatitis B virus variants carrying in-frame deletions in the central part of the core gene [19].

It is known that variants with in-frame deletions in the central region of the core gene are usually present together with the wild-type virus [5]; therefore, it is likely that presence of the intact core protein might be needed in case mutant proteins are unable to form functional core particles. Also, the accumulation of mutant proteins is frequently accompanied by the inhibition of wt virus replication [32]. Since HBV core protein (HBc) deletion variants are rapidly degraded via the proteasomal pathway [7], the ability of some mutant proteins to interact with the wild-type HBc protein might contribute to this inhibition. To clarify these issues, wt and seven internally deleted core proteins, isolated from kidney-transplant patients in Germany [38], were tested for their ability to interact with each other in the yeast two-hybrid system.

The accumulation of described HBV mutants frequently leads to the development of HBV-related end-stage liver disease and death of renal transplant recipients. However, it still remains unclear how the mutated viruses invoke the progression of liver disease. Analyzed mutated viruses differ from their wt counterpart only by deletions inside the HBc gene; therefore, liver disease might be the result of direct cytotoxicity caused by the internally deleted HBc proteins. Mutant proteins of severely mutated viruses, incapable to survive under normal conditions, might cause damage to the host cell by interacting with proteins unrelated to virus survival. Identification of such interactions could contribute to understanding the mechanisms of viral pathogenesis. To find host proteins possibly involved in enhanced pathogenesis of the mutant HBV variants, a human hepatocyte cDNA library was screened for proteins interacting with the mutant but not with the wt core protein.

Although HBV is relatively well-studied, little is known about its core protein interactions with host proteins. There are emerging evidences, however, that structural proteins can play a significant role in viral pathogenesis. For example, human hepatitis C virus core protein is involved in apoptosis, immunomodulation, oxidative stress, carcinogenesis, and other processes [22]. In order to enlighten the role of viral structural proteins in the pathogenesis of HBV-infected hepatocytes, it is necessary to investigate their interactions with host cell proteins. Discovered interactions might be helpful in

identifying human proteins participating in important stages of the virus life cycle, such as virus entry and transport of nucleocapsids to the nucleus. Established protein contacts could serve as targets for antiviral chemotherapy. Therefore, in this work a human liver cDNA library was screened also for proteins interacting with wt HBV core protein.

**The aims of this study were:**

1. To find human liver proteins interacting with HBV core protein.
2. To find human liver proteins interacting with HBc deletion variants isolated from highly pathogenic HBV mutants.
3. To characterize discovered interactions as well as interactions between HBc protein and its deletion variants.

**Scientific novelty:**

Several new HBc-interacting partners revealed. Human proteins GIPC1 and GIPC2 interacted most strongly and specifically with both mutant and wt HBc proteins. Common protein interaction domain PDZ was identified as the region, sufficient for discovered interactions in both proteins. A putative PDZ-interacting motif detected inside the core protein, and this sequence proved to be important for the interaction with GIPC1 and GIPC2. Several human proteins interacted with HBc deletion variants only. The gene and expression pattern of protein with unknown function FLJ20850 was characterized by bioinformatics methods. An attempt to determine interacting regions of both proteins revealed that FLJ20850 was unable to interact without significant parts of its C- or N-end, and introduced deletion in the central region conferred interaction capability to the wt core protein. Interaction of HBc deletion variants with another human protein, IKK $\gamma$ , was also analyzed in detail, and interacting region of this protein was determined.

# MATERIALS AND METHODS

## Chemicals and enzymes

All reagents used in this study were reagent-grade commercial products. Nutrient media components were purchased from *Carl Roth GmbH* (Karlsruhe, Germany), salts for preparations of buffers were from *Amresco Inc.* (Solon, OH, USA). *Nucleospin Plasmid* (*Macherey-Nagel*, Duren, Germany) and *GeneJET Plasmid Miniprep Kit* (*UAB Fermentas*, Vilnius, Lithuania) were used for purification of plasmid DNA. *Cyclo-pure gel extraction kit* (*Amresco*) was used for purification of DNA from agarose gels. *BigDye® Terminator v3.1 Cycle Sequencing kit* was from *Applied Biosystems*. Glutathione-agarose beads, IPTG, *pGex-5x* plasmid, IPTG and anti-GST antibody were obtained from *Amersham Biosciences* (Piscataway, NJ, USA). Anti-HA monoclonal antibodies were obtained from *Sigma-Aldrich Co.* (Natick, MA, USA). DNA and protein molecular weight markers, all enzymes, their reaction buffers and kits were purchased from *UAB Fermentas* and used according to the manufacturer's recommendations.

## Bacterial and yeast strains

### *Escherichia coli*:

- DH5 $\alpha$  F-( $\phi$ 80dlacZ  $\Delta$ M15) recA1 endA1 gyrA96 thi-1 hsdR17(r<sub>k</sub>-m<sub>k</sub><sup>+</sup>) supE44 relA1 deoR  $\Delta$ (lacZYA-argF) U169;  
BL21 F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) dcm gal (DE3);  $\lambda$ DE3 lysogen containing the T7 RNA polymerase gene (*Novagen*);  
KC8 hsdR, leuB600, trpC9830, pyrF::Tn5, hisB463, lac $\Delta$ X74, strA, galU, K (*Clontech*).

### *Saccharomyces cerevisiae*:

- EGY48 MAT $\alpha$ , ura3, his3, trp1, LexAop(x6)-LEU2 (*Clontech*).

## Plasmids

- pUC57 for cloning of PCR fragments (*Fermentas*);  
pTZ57R/T for cloning of PCR fragments (*Fermentas*);  
pGex-5x for construction and bacterial expression of GST (26-kDa glutathione-S-transferase domain from *Schistosoma japonicum*) fusions (*Amersham*);  
pET-HA for construction and bacterial expression of HA (influenza hemagglutinin-HA epitope) tagged proteins (constructed by the authors);  
pHB320 contains full-length HBV ayw genome (prof. Paul Pumpens, Biomedical Research and Study Center, University of Latvia, Riga, Latvia);  
pC19 contains HBc gene with 86-93 amino acids (aa) deletion (dr. Helga Meisel Institute of Virology, Charité University Hospital, Berlin, Germany);  
W1207co contains HBc gene with 77-93 aa deletion (dr. Helga Meisel);



pLexA	for construction and expression of LexA-bait fusions for two-hybrid experiments ( <i>Clontech</i> )
pB42AD	for construction and expression of B42-prey fusions for two-hybrid experiments ( <i>Clontech</i> );
p8op-lacZ	reporter plasmid in two-hybrid experiments contains $\beta$ -galactosidase gene under the control of LexA operator ( <i>Clontech</i> );
pB42AD-T	negative control plasmid, SV40 virus large T antigen fused with b42 domain in pB42AD ( <i>Clontech</i> );
pLexA-Lam	negative control plasmid, human lamin C fused with lexA domain in pLexA ( <i>Clontech</i> ).

## Oligonucleotides

### *Sequencing primers (Fermentas):*

M13/pUC dir	5'-GTAAAACGACGGCCAGT
M13/pUC rev	5'-CAGGAAACAGCTATGAC
T7-prom	5'-TAATACGACTCACTATAGGG
T7-term	5'-GCTAGTTATTGCTCAGCGG

### *Sequencing and amplification primers for inserts inside pB42AD (Clontech):*

AD-dir	5'-CGATACCAGCCTCTTGCTGAGTGGAGATG
AD-rev	5'-GATTGGAGACTTGACCAAACCTCTGGC

### *Oligonucleotides for NF- $\kappa$ B detection by EMSA (Metabion)*

oNF $\kappa$ B1	5'-CGAGCCTAACGGGACTTTCCAAG
oNF $\kappa$ B2	5'-TCGAGCTCGGATTGCCCTGAAAGGTTTCAGCT

### *Oligonucleotides for construction of plasmids (Metabion, MWG Biotech):*

QMN1	5'-GATCCCAATTGCCCGGGCCCGTCGACCTGCA
QMN2	5'-GGTCGACGGGCCCCGGGCAATTGG
HAP1	5'-CTAGACCACCATGGTATACCCATACGATGTTCCAGATTACGCTG
HAP2	5'-TGGTGGTACCATATGGGTATGCTACAAGGTCTAATGCGACCTAG
HAP3	5'-GATCCAGCGTAATCTGGAACATCGTATGGGTATACCATGGTGGT
2H1	5'-GCCAATTCGACATTGATCCTTATAAAGAA
2H2	5'-GCCTCGAGCTAACATTGAGATTCCCGAG
CON1	5'-ACCATGGACATTGATCCTTATAAAGAATTTG
CoreDD2	5'-GGCCTAAAATTCAGGCAACTATTG
2053	5'-ATAACTGACTACTAGGTCCCTG
2054	5'-GTTGACATAACTGACTACTAGGTCC
CCS2A	5'-GCCTCGAGCTAACATTGAGCTTCCCGAG
CCC0A	5'-GCCTCGAGCTAAGCTTGAGATTCCCGAG
CCD1	5'-TGGAATTCCCGGAGACTACTGTTGTTAGAC
CCR1	5'-CTCGAGCTAGCGCGCATTTGGTGGTCTATAAGC
CCR2	5'-CTCGAGCTAGCGCGCTTGAGATCTTCTGCGACGCG
2051	5'-CACTCGAGTTACACTGGCCGGCCCTACTC
IKKD2	5'-GGAATTCAGGAAGCTGGCCCAGTTGCAG
IKKD3	5'-GGAATTCGTGGGCAGTGAGCGGAAGCG

IKKD4	5'-GGAATTCCAGGCGGATATCTACAAGGCG
IKKR1	5'-GCTCGAGCTACGCCTGGGCCTTCAGCACCG
IKKR2	5'-CCTCGAGCTACCGCTTCCTCATGTCTCG
GC1R1	5'-GCTCGAGCTAGTAGCGGCCGACCTTGG
GC1D1	5'-GGAATTCTGAAGAGAAGGCCATTGAGAAGG
GC1D2	5'-GGAATTCTGCCACGTGAAGGGGCAG
GC1R2	5'-GCTCGAGCTAGCCACCCGCTGAACGCTGGC
GC2R1	5'-CTCGAGTCATAATCCTCTTCGTTTGGC
GC2D1	5'-GGAATTCGGTCCTGCCACCGTGGAAG
GC2D2	5'-GAATTCGGAGGACAAGTAGGACTAGAAG
GC2R3	5'-CTCGAGTCACTTCATAGTAAAGAGTTCCTC
A1R1	5'-CTCGAGCCGCCATCACTCGGCAG
A1D2	5'-GAATTCGCGCGCTACCGGAGC
A1R2	5'-CTCGAGTCAGCCCAGCCGCGTCTGCTCC
A1D4	5'-GAATTCCTGCAGTGCTGCTGC

### DNA preparation and manipulation

DNA digestion with restriction endonucleases, DNA ligation, dephosphorylation, polymerization reactions were performed according to manufacturer's recommendations (*Fermentas*). DNA electrophoresis was carried out according standard technique [47]. Plasmid DNA preparation was carried out using alkaline lysis method [2, 47].

### Construction of recombinant plasmids

#### *pLexA-HBc, pB42AD-HBc, pGex-5x-HBc ant pET-HA-HBc*

The full length HBc gene was cloned into plasmid pTZ57R/T after PCR amplification with primers 2H1 and 2H2. After sequencing and digesting with EcoRI and XhoI, the fragment was inserted into the appropriately digested plasmids pLexA, pB42AD, pGex-5x and pET-HA, suitable for yeast and bacterial expression. Plasmids for expression of all other amplified fragments were constructed identically to plasmids for expression of the full length core gene.

#### *Plasmids with altered HBc genes*

The HBc gene containing alanine in 181 position instead of serine was amplified with primers 2H1 and CCS2A; cysteine in 183 position was replaced with alanine by amplifying HBc gene with primers 2H1 and CCC0A; a fragment encoding 144-183 aa of the HBV core protein was amplified with primers CCD1 and 2H2; a fragment encoding 1-177 aa of the core protein was amplified with primers 2H1 and CCR2. Plasmids containing hybrid Wt-c1 and c1-Wt genes were constructed by digestion of core genes with *Kpn2I* and *XhoI* and replacing 3' fragment of one gene with the fragment of another gene. Core gene with deletion of 5 amino acids Wt-5 was constructed by amplifying plasmid containing inserted wt core with primers CDD and CD5R and by circularizing the resulting fragment.

### *GIPC1 gene alterations*

A DNA fragment encoding 124-228 aa of GIPC1 was amplified with primers GC1D2 and GC1R2; a fragment encoding 124-333 aa of GIPC1 was amplified with GC1D2 and GC1R1; a fragment encoding 261-333 aa was amplified with GC1D1 and GC1R2.

### *GIPC2 gene alterations*

A DNA fragment encoding 98-315 aa of GIPC2 was amplified with primers GC2D2 and GC2R1, a fragment encoding 98-194 aa of GIPC2 was amplified with GC2D2 and GC2R3; a fragment encoding 231-315 aa was amplified with GC2D1 and GC2R1.

### *Truncated forms of FLJ20850 gene*

*FLJ20850* gene containing 12-201 aa was amplified with primers A1D4 and A1R1; 12-150 aa fragment amplified with A1D4 and A1R2; 97-201 aa fragment obtained by amplification with A1D2 and A1R1; 97-150 aa fragment amplified with A1D2 and A1R2.

### *IKK $\gamma$ gene alterations*

IKK $\gamma$  225-419 aa fragment obtained by amplifying IKK $\gamma$  gene with primers IKKD2 and 2051; 250-419 aa fragment amplified with IKKD3 and 2051; 250-305 aa fragment amplified with IKKD3 and IKKR1; 304-419 aa fragment amplified with IKKD4 and 2051; 250-359 aa fragment amplified with IKKD3 and IKKR2.

## **Assay for reporter activity in yeast cells**

Eight individual EGY48 clones transformed with the same bait-prey plasmid combination were spotted on plates with SD medium and, after overnight incubation at 30 °C, replica-plated on SD/Gal medium prepared in PBS (pH 7.0) with 0.1 mg/mL of X-gal and on SD/Gal/-Leu medium. After incubation at 30 °C for 24-48 h, yeast cells with interacting protein hybrids developed blue color and were able to grow on medium without leucine. To perform  $\beta$ -galactosidase liquid assay, yeast were grown on inductive medium, resuspended in 300  $\mu$ l of disruption buffer (Z-buffer (100 mM NaPi, 10 mM KCl, 1 mM MgSO<sub>4</sub>, pH 7) with 0.2 mM PMSF and 0.3%  $\beta$ -mercaptoethanol), frozen in liquid nitrogen, and disrupted with glass beads. Then 100  $\mu$ l of each lysate was supplemented with 700  $\mu$ l of disruption buffer and 160  $\mu$ l of ONPG solution (4 mg/ml in Z buffer). The reaction was terminated by adding 0.5 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> after yellow color developed. The incubation time and A<sub>420</sub> were recorded. Results within the linear range were expressed as  $\mu$ Mol of ONPG hydrolyzed by 1 mg of protein per minute. To enhance data reliability, four separate transformants were assayed, each in triplicate.

## **Yeast transformation and two-hybrid library screening**

Growth, transformation and selection of positive yeast clones were performed according to *Clontech* protocols. To group colonies with identical cDNA inserts, PCR was performed with primers ADdir and ADrev, and amplified fragments were digested with four nucleotide-specific restriction enzymes. Yeast plasmid DNA was isolated using the lyticase method and transformed into E.coli strain KC8.

## **Protein expression in bacteria, electrophoresis and Western blotting**

Transformed *E. coli* strain BL21 (DE3) was grown at 37 °C to an A600 of 0.6 in 200 mL of LB medium containing 50 mg/L of ampicillin. After addition of 1mM of IPTG, the culture was shaken at room temperature for another 16 h. The cells were harvested, resuspended in 3 mL of PBS buffer (pH 7.2) with 2 mM PMSF, 1 mM EDTA, and 3 g of glass beads (0.5 mm diameter), then disrupted by vortexing at 4 °C for 4 min. Denatured cell extracts were subjected to electrophoresis in 12-15% SDS-polyacrylamide gels and stained with Coomassie Blue or transferred to PVDF membranes. The membranes were blocked with 5% of nonfat dry milk for 1 h and incubated with the monoclonal anti-HA or anti-GST antibody for another 2 h. Antibody-reactive proteins were detected with a HRP-conjugated secondary antibody after 1h of incubation.

## **In vitro binding assay**

Bacterial lysates with expressed GST fusion proteins were allowed to bind 25 µL of glutathione-agarose beads for 1h at 4 °C. After incubation, the beads were washed three times with PBS (pH 7.2) at 4 °C, supplemented with bacterial lysates containing expressed HA-tagged proteins, then beads incubated for 4 h at 4 °C to allow protein-protein interactions to occur. The beads were washed four times with 0.7 mL of PBS, and resin-bound proteins were resolved on SDS-polyacrylamide gels. After being transferred to PVDF membranes, proteins were detected with anti-HA or anti-GST antibodies.

## **NF-κB activity test by measuring CAT reporter gene activity**

Human granulocyte-monocyte progenitor cell line HL-60 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% humidified atmosphere. Plasmid pRR55 with chloramphenicol acetyltransferase (CAT) gene fused to human cytomegalovirus (HCMV) immediate-early (IE) enhancer/promoter and plasmids for expression of HBc genes were transfected by the DEAE dextran method [47]. Half of the cells were supplemented with 5 ng/ml of TNF-α 2 h before disruption. The cells were then washed with PBS and disrupted by repeated freezing and thawing. After centrifugation, samples of the supernatants were assayed for protein content. Equal quantities of protein were incubated with [14C]chloramphenicol (*Amersham*); the acetylated products were quantified after separation by thin-layer chromatography using a thin layer scintillator. Promoter activities were estimated relative to the conversion rate of [14C]chloramphenicol under the influence of cytokines and compared with the control samples.

## **NF-κB activity test by electrophoretic mobility shift assay**

Human hepatocyte cell line Huh7 was transfected with plasmids for expression of HBc genes employing *Effectene* (*Qiagen*) or *ExGen 500* (*Fermentas*) transfection reagents. Transfected cells were grown overnight in plates with diameter of 6 cm, and 2 h before disruption half of them supplemented with 5 ng/ml of TNF-α. Cells washed with ice-cold PBS, resuspended in 1,5 ml buffer A (100 µl HEPES (1M, pH 7,9), 100 µl KCl (1M), 10 µl EDTA (0,1 M), 10 µl EGTA (0,1 M), 100 µl DTT (10 mM), 50 µl PMSF (10 mM), water to 10 ml) for 15 min., then buffer A with 10% of *Triton X* was added, and cells lysed by

shaking for 5 min. Cell lysates were centrifuged, washed with 2-3 mL of buffer A, resuspended in 100  $\mu$ L of buffer B (20  $\mu$ L HEPES (1M, pH 7,9), 133  $\mu$ L KCl (3M), 1  $\mu$ L EDTA (0,1 M), 1  $\mu$ L EGTA (0,1 M), 10  $\mu$ L DTT (10 mM), 10  $\mu$ L PMSF (10 mM), water to 1 ml), shaken for 30 min. at 0-4  $^{\circ}$ C, and nuclear protein extract separated by centrifugation. Each sample with 10  $\mu$ g of nuclear proteins was supplemented with 20  $\mu$ L EMSA reaction buffer (10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM spermidin, 10% glycerin), containing 1,2  $\mu$ g of poly(dI-dC) and 0,5-1 ng P<sup>32</sup>-labeled  $\alpha$ NF $\kappa$ B1- $\alpha$ NF $\kappa$ B2 oligonucleotides mix. After 20 min. of incubation in room temperature, reaction products were separated in 5% non-denaturing PAGE.

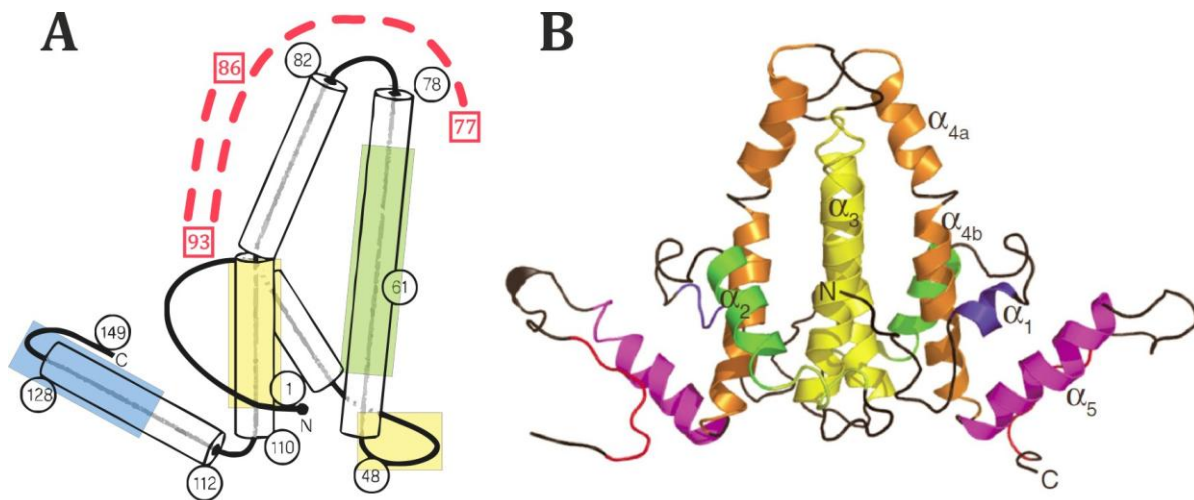
### **DNA sequencing and bioinformatics**

cDNA inserts and constructed plasmids were sequenced in Sequencing Center of Institute of Biotechnology (Vilnius, Lithuania) with *BigDye® Terminator v3.1 Cycle Sequencing Kit* and *Gene Analyzer 3130xl (Applied Biosystems)*. Homology searches for the DNA and deduced amino acid sequences were performed by using the BLAST network service at NCBI. Search for protein motifs was performed by EMBL SMART and Welcome Trust Sanger Institute's PFAM web services. Prediction of exon-intron structures in genomic sequences was performed by GENSCAN web server at MIT. Prediction of coiled-coil regions in proteins was carried out on Coils server, and multiple protein alignment was accomplished by Toffee server, both hosted by Swiss Institute of Bioinformatics.

## RESULTS AND DISCUSSION

### Interaction between wild-type and mutant HBV core proteins in the yeast two-hybrid system

The accumulation of hepatitis B virus variants carrying in-frame deletions in the central part of the core gene frequently leads to the development of severe forms of hepatitis [19]. Variants with in-frame deletions in the central region of the core gene are present together with the wild-type virus [5], and their accumulation is accompanied by inhibition of wt virus replication [32]. Since HBc deletion variants are rapidly degraded via the proteasomal pathway [7], the ability of some mutant proteins to interact with the wild-type HBc protein might contribute to the inhibition of the wt virus due to formation of defective or rapidly degraded mosaic particles. The presence of the wt virus might be necessary if the mutant core proteins are incapable to form functional particles. To test these assumptions, properties of some HBc deletion mutants were already analyzed in bacterial and human cells. It was determined that only few deletion mutants were able to form particles consisting of mutant proteins only and mosaic particles consisting of mutant and wt protein. Some core mutants in these systems were incorporated only into the mosaic particles [38]. In the present work, the wt (**Fig. 1**) and mutant core proteins (**Table 1**), isolated from kidney-transplant patients in Germany [38], were tested for their ability to interact with each other in the yeast two-hybrid system.



**Figure 1. The structure of HBV core protein.** **A.** Diagram of truncated (149 aa) HBc monomer. The  $\alpha$ -helical regions are indicated as cylinders. Numbers inside the circles indicate positions of important amino acids: N- and C-ends, ends of  $\alpha$ -spirals, and Cys61, which participates in dimeric junction. Red dashed lines indicate deletions of HBc1 and HBc2 mutants. Colored areas: yellow, essential for forming of the tertiary structure; green, areas participating in dimer formation; blue, essential in interaction between dimers. According to Bottcher *et al.* [6]. **B.** The structure of HBc 1-149 dimer. The  $\alpha$ -helices of each monomer are colored and graded from the N-terminus through the C-terminus (blue running through red, respectively). Helices span the following residues:  $\alpha_1$  (13–17),  $\alpha_2$  (27–43),  $\alpha_3$  (50–73),  $\alpha_{4A}$  (79–90),  $\alpha_{4B}$  (92–110), and  $\alpha_5$  (112–127).

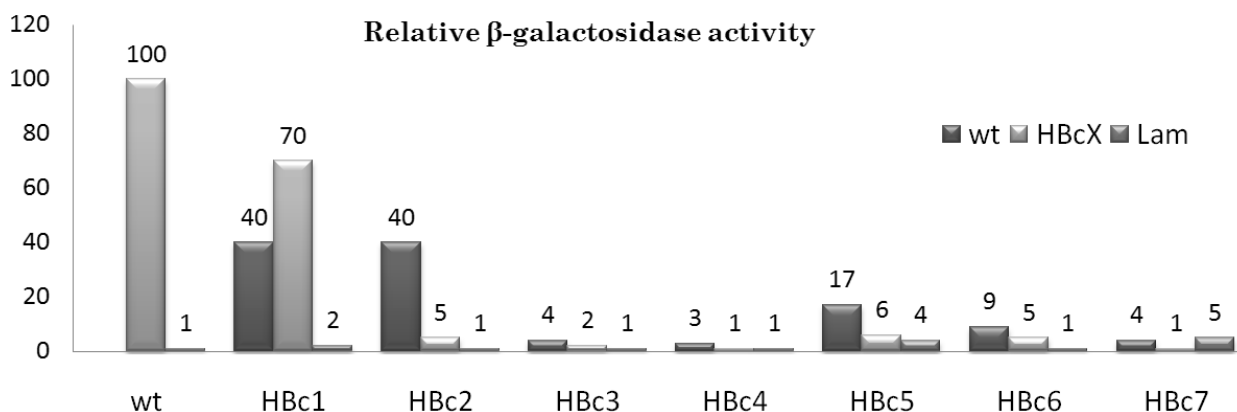
The wild-type core protein was able to interact with the wt as well as with the deletion variants HBc1 and HBc2 (**Fig. 2**). The wild-type protein also weakly interacted with

another two mutants – HBc5 and HBc6. These results support the possibility that mutant core variants might contribute to observed wild-type virus inhibition by forming dimers with the wt HBC proteins. This possibility is also supported by the observation that core gene promoter mutations compensate the suppression of wt virus by core gene deletion variants [32]. It is also obvious that mutant proteins (except HBc1) are unable to form homodimers – a prerequisite for the formation of the intact core particle. Therefore, this experiment also supports the assumption that the presence of wt HBV virus is required by most mutant viruses to provide viable core particles, necessary for the completion of virus replication cycle.

**Table 1. HBV core deletion mutants**

Mutant	HBc1	HBc2	HBc3	HBc4	HBc5	HBc6	HBc7
<b>Deletion</b>	86-93	77-93	82-111	75-95	45-46, 86-93	85-93, 102-113	45-51
<b>Diagnosis</b>	ESLD	ESLD	ESLD	ESLD	ESLD	CH	ESLD

**Abbreviations:** *ESLD*, end stage liver disease; *CH*, chronic hepatitis. Number range indicates deleted amino acids.



**Figure 2. Interactions of native and mutant HBV core proteins in the yeast two-hybrid system as determined by β-galactosidase liquid assay.** Data represent interactions of core deletion mutants (HBc1–HBc7) with the wild-type HBC (wt), the same mutant (HBcX), and control protein human lamin (Lam). All β-galactosidase activity data are relative to wt-wt interaction.

## Search for human proteins, interacting with wild-type and mutant HBV core proteins

It still remains unclear how the mutated HBC proteins are involved in the progression of liver disease. One possible mechanism could be a direct cytotoxicity caused by the internally deleted HBC proteins. Whereas the purpose of pathological interactions usually is to weaken host antiviral responses or secure host resources for viral replication, mutant proteins of severely mutated viruses might cause unexpected damage to the host cell by interacting with proteins unrelated to virus survival. Identification of such interactions could be helpful in understanding the mechanisms of viral pathogenesis. In the same time, discovered unknown interactions with the wt HBC might be helpful in identification of human proteins participating in important stages of the virus life cycle. Established protein contacts could serve as targets for antiviral chemotherapy.

**Table 2. Human proteins interacting with wild-type and mutant HBc proteins**

Protein	cDNA completeness	Protein description
<b>GIPC1</b>	Missing 60 aa in N-end; C-end complete; 273 aa total. Two cDNAs sequenced.	Connects other proteins with the movement of myosin VI along actin filaments. More detailed description of this protein is presented in the next chapters.
<b>GIPC2</b>	N-end lacks 48 aa; C-end complete; 267 aa total. One cDNA clone sequenced.	Function unknown, significant homology with GIPC1. More detailed description of this protein is presented in the next chapters.
<b>RPL5</b>	N-end lacks about 100 aa; C-end complete; total length 181 aa. Two cDNA clones sequenced.	L5 is the main component of 5S ribosomal RNA transporter from the nucleus. L5 interacts with eukaryotic translation initiation factor 5A, the protein interacting with HIV protein Rev and participating in viral mRNA transport across the nuclear envelope. Increased L5 synthesis accelerates, and inhibition of the synthesis stops viral mRNA transport and HIV replication [48]. HBc, which binds viral genome and can bind other RNAs, might perform similar functions by interacting with L5. L5 also implicated in oncogenic transformation and forms complexes with Mdm2 and p53.
<b>Fibrinogen <math>\gamma</math></b>	N-end lacks 315 aa; C-end complete; total length 122 aa. Two cDNA clones sequenced, five selected.	Wild-type HBc interacts stronger with $\gamma$ - and $\alpha$ -fibrinogens than the mutant proteins. Fibrinogens are soluble plasma glycoproteins, synthesized in the liver, and converted into fibrin during blood coagulation. It is known, that viral hepatitis patients frequently suffer from abnormalities of fibrin formation and blood clotting [11]. Most likely, the main cause of these deficiencies is the damage of the liver. However, detected interaction of HBc with fibrinogens could contribute to this process, especially because blood of HBV infected patients contains large quantities of another form of the core protein – antigen e (HBeAg).
<b>Fibrinogen <math>\alpha</math></b>	N-end lacks 86 aa; C-end complete; total length 558 aa. One cDNAs clone sequenced, four selected.	The same as for the fibrinogen $\alpha$ protein.

To find such interacting partners, yeast expressing the HBc1 fusion protein were transformed with human adult liver cDNA library. Nearly three millions of transformed cells were plated on medium lacking leucine, and after three days growing colonies were tested for  $\beta$ -galactosidase reporter activity. Both reporter genes were active in more than one hundred of colonies. After transformation with plasmids expressing negative control proteins and reporter gene activation test, false-positive or weakly positive clones were discarded. The second round of screening was performed by testing the selected human proteins for their ability to interact with wt HBc. Less than half of the fifty selected clones were able to interact with wt HBc. All plasmids with cDNA clones positive for interaction with HBc1 were also positive for interaction with HBc2 mutant.

Thus, all selected clones were divided into two groups: those interacting with both wt and mutant (HBc1 and HBc2) proteins and those interacting with mutant (HBc1 and



Hbc2) proteins only. Twenty two plasmids from clones interacting with wt and mutant proteins were sequenced, and BLAST analysis identified selected sequences as human cDNAs for GIPC1, GIPC2, ribosomal protein L5, and fibrinogen polypeptides alpha and gamma (**Table 2**). Thirty plasmids with cDNA clones specific to mutant proteins only were identified as cDNAs of motor and regulatory proteins mainly: kinesin family member 13A, NOTCH2NL, fumarate hydratase, IKK $\gamma$ /NEMO, and proteins with unknown functions CASKIN2 and FLJ20850 (**Table 3**).

**Table 3. Human proteins interacting only with mutant Hbc proteins**

Protein	cDNA completeness	Protein description
<b>IKK<math>\gamma</math>/NEMO</b>	Missing 224 aa in N-end; C-end complete; 195 aa total. One cDNA clone sequenced.	Protein of NF- $\kappa$ B signaling pathway. More detailed description of this protein is presented in the next chapters.
<b>FLJ20850</b>	N-end lacks 8 aa; C-end complete; 171 aa total. One cDNA sequenced.	Protein of unknown function. More detailed description and analysis of this protein is presented in the next chapters.
<b>KIF13A</b>	Cloned cDNA encodes 1757 aa of the N-end of KIF13A protein with kinesin motor domain (~600 aa). N-end lacks 195 aa. One cDNA clone sequenced.	Protein of kinesin family participating in the peripheral cell transport [13]. Kinesins are motor proteins moving along microtubules by hydrolyzing ATP. Viral genomes and protein complexes require active transporting in the cytoplasm, and for this purpose they employ host motor proteins [14]. Viral proteins interacting with molecular motors can disrupt important cellular transport systems or can be delivered to the sites where they cause their pathogenicity.
<b>NOTCH2NL</b>	N-end lacks 124 aa; C-end complete; total length 112 aa. One cDNA sequenced.	Human NOTCH2NL (Notch homolog 2 N-terminal like) [15] is the member of the Notch signaling pathway [51]. Mutations of genes and other abnormalities of this pathway are associated with leukemia, breast, prostate, cervix and other cancers [42]; this protein is implicated in Alzheimer disease [55]. Members of the Notch family also interact with viral proteins. Interaction of Epstein-Bar virus EBNA2 protein with Notch1 and Notch2 leads to oncogenic transformation of B lymphocytes; members of the Notch family interact with Kaposi sarcoma and human papilloma viruses [20]. NOTCH2NL protein is described recently and so far is implicated only in inhibiting the genes of the Notch family.
<b>Fumarase</b>	N-end lacks 199 aa; C-end complete; total length 311 aa. One cDNA sequenced.	Fumarase (fumarate hydratase) is enzyme of the Krebs cycle. Fumarase deficiency is a rare recessive autosomal disease, causing serious neurological and liver damage. Fumarase mutations are associated with various cancers [18].
<b>CASKIN2</b>	N-end lacks 863 aa; C-end complete; total length 257 aa. One cDNA sequenced.	CASKIN2 (cask-interacting protein 2) described only recently [49]. CASK protein is involved in a pathway transmitting signals from the synapse [21] but its interaction with CASKIN2 is not determined. CASKIN2 received its name because of homology with CASKIN1, which interacts with CASK. So far, CASKIN2 functions and interactions with other proteins are unknown.

Interacting specificity of each selected protein was further evaluated by measuring interactions between various human proteins selected during two-hybrid screen. Additional experiments revealed that most identified cDNAs, although unable to interact with standard controls, interacted with some other selected cDNAs, thus they were regarded as having low interaction specificity. Interactions of wt or mutant core proteins with human GIPC1, GIPC2, FLJ20850, and IKK $\gamma$ /NEMO were strongest and most specific, and these cDNAs were selected for further analysis.

## **Human proteins interacting with wild-type and mutant HBc proteins**

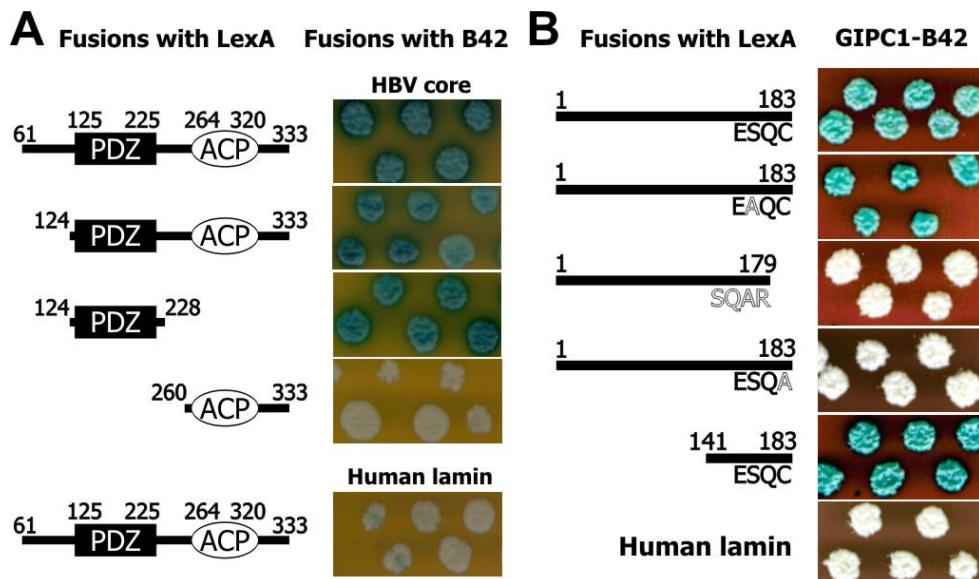
### **GIPC1**

GIPC1 (GAIP interacting protein, C terminus, also known as GIPC, TIP-2, GLUT1CBP, RGS19IP1, synectin, SEMCAP, NIP, and IIP-1) is a human protein interacting with several cellular proteins, as well as with viral proteins [12]. In most cases these interactions are performed by a PDZ domain, which initially was identified by uniform sequence repeats in PSD-95, Dlg, and a tight junction protein ZO-1 [27]. PDZ domains are built of 80–100 amino acid residues specialized for binding of C-termini in partner proteins, most often transmembrane receptors and channel proteins, or other PDZ domains [25]. The two-hybrid screen resulted in nearly full-length GIPC1 cDNA fragments with intact PDZ domain. To test whether the PDZ domain is involved in the interaction with HBc, two-hybrid plasmids containing the PDZ domain only (residues 124–228), PDZ domain with adjacent C-terminal region, and C-terminal region only were constructed. GIPC1 61–333, GIPC1 124–333, and GIPC1 124–228 fusions with LexA were able to interact with the core protein–AD domain fusion, whereas GIPC1 260–333 did not interact (**Fig. 3, A**). These results imply that the PDZ domain is responsible for the interaction with the HBV core protein.

PDZ domains usually interact with three or four C-terminal amino acids of their protein partners; therefore, it was presumed that GIPC1 interacts with the C-terminus of the core protein. To test this hypothesis, two-hybrid plasmid containing 141–183 amino acids of HBc was constructed. Experiments indicated that the C-terminus of the core protein is sufficient for the interaction with GIPC1 (**Fig. 3, B**). To determine whether terminal amino acids of HBc are indeed important for the interaction with GIPC1, four C-terminal residues were removed. The altered C-terminus (Ser-Gln-Ala-Arg-COOH) is not similar to any known PDZ binding motif (PBM), and such modified core protein was unable to interact with GIPC1 (**Fig. 3, B**).

According to their binding selectivity, PDZ domains have been placed into three classes. Class I domains recognize the consensus sequence Ser/Thr-X- $\Psi$ -COOH (where X is any amino acid and  $\Psi$  is hydrophobic); class II domains prefer  $\Psi$ -X- $\Psi$ -COOH; class III domains prefer Asp/Glu-X- $\Psi$ -COOH [36]. Initially, the specificity of GIPC1 PDZ domain was considered to be class I [53]. In some cases substitution of serine or threonine with another amino acid in the -2 position of PBM interfered with GIPC1 binding [50]. (By convention, the C-terminus of PDZ ligands is designated as residue 0, and subsequent residues toward the N-terminus are numbered as residue -1, residue -2, and so forth [1].) But replacement of -2 serine with alanine did not prevent HBc from interaction

with GIPC1 (**Fig. 3, B**). The C-terminus of such modified core protein (Glu-Ala-Gln-Cys-COOH) is similar to the termini of another GIPC1-interacting proteins—dopamine receptors D2 (Ile-Leu-His-Cys-COOH) and D3 (Ile-Leu-Ser-Cys-COOH) [24]—both these proteins contain leucine instead of serine or threonine in the -2 position. When more data accumulated, it became clear that PDZ domain of GIPC1 binds sequences of all three classes [41]. But in slightly altered classification, class III binding motif is designated as X-X-Cys-COOH (**Table 4**). Both the C-terminal sequence of HBc Glu-Ser-Gln-Cys-COOH and the modified sequence Glu-Ala-Gln-Cys-COOH resemble this motif. Modified HBc with alanine instead of cysteine in C-terminal position was unable to interact with GIPC1 (**Fig. 3, B**).



**Figure 3. Interaction between GIPC1 and HBV core protein in the yeast two-hybrid system. A:** Determination of GIPC1 interacting region. Truncated forms of GIPC1 protein and human lamin C (negative control) probed for interaction with HBc. **B:** Mapping of HBc interacting region. Nearly full-length GIPC1 (61–333 aa) was probed for interaction with various modifications of HBc and human lamin C.

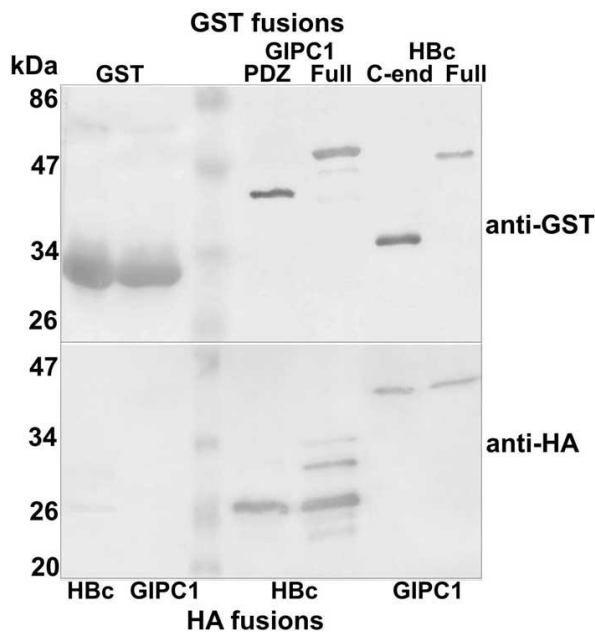
The GST pull-down in vitro assay was used to confirm the determined protein interactions. Various fragments of HBV core and GIPC1 were inserted into bacterial expression vector containing GST gene. To enable uniform detection of interacting proteins, the same sequences were fused with the short coding sequence of influenza virus hemagglutinin HA epitope. Proteins containing GST or HA on their N-terminus were synthesized in bacteria at different quantities, their solubility also varied. Native GST protein, typical negative control in such type of experiments, was more soluble and bound more efficiently to glutathione beads than to any of the fusion proteins. GST and its fusions with full-length HBc or HBc 141–183 were immobilized on glutathione beads, and tested for their ability to interact with HA-tagged GIPC1 61–333. After washing, the same amounts of beads were analyzed for the presence of the tagged proteins, and GIPC1 was found only on the beads with core-containing fusion proteins. Similarly, GST fusions with GIPC1 124–228 and GIPC1 124–333, but not GST alone, were able to bind HA-tagged full-length core protein (**Fig. 4**). These results confirm that the PDZ domain of GIPC1 interacts with the C-terminus of the HBV core protein.

**Table 4. Sequences recognized by the GIPC1 PDZ domain**

<b>Interacting protein</b>	<b>C-end sequence*</b>
<b>I class PBM</b>	<i>Ser/Thr-X-Ψ-COOH</i>
<b>GLUT1</b>	...Asp-Ser-Gln-Val
<b>KIF-1B</b>	...Glu-Thr-Thr-Val
<b>α-Actinin</b>	...Glu-Ser-Asp-Leu
<b>RGS-GAIP</b>	...Ser-Ser-Glu-Ala
<b>Neuropilin</b>	...Tyr-Ser-Glu-Ala
<b>SemF</b>	...Glu-Ser-Ser-Val
<b>SemC</b>	...Asp-Ser-Val-Val
<b>GP75(Trp-1)</b>	...Glu-Thr-Glu-Val
<b>Megalyn (LDLR)</b>	...Gln-Ser-Val-Val
<b>5T4</b>	...Asp-Ser-Glu-Val
<b>Integrin α6A</b>	...Xxx-Ser-Asp-Val
<b>Integrin α6B</b>	...Thr-Ser-Asp-Ala
<b>TGF-βIII receptor</b>	...Glu-Ser-Tyr-Ser
<b>α-Adrenergic receptor</b>	...Ser-Ser-Thr-Ala
<b><i>HIV-1 Tax</i></b>	<i>...Glu-Ser-Lys-Val</i>
<b><i>HPV-16 E6</i></b>	<i>...Glu-Thr-Gln-Leu</i>
<b>II class PBM</b>	<i>Ψ-X-Ψ-COOH</i>
<b>Syndecan-4</b>	...Glu-Phe-Tyr-Ala
<b>Syndecan-2</b>	...Glu-Phe-Tyr-Ala
<b>III class PBM</b>	<i>X-X-Cys-COOH</i>
<b>IGF-1 receptor</b>	...Ser-Ser-Thr-Cys
<b>Human LH receptor</b>	...Tyr-Thr-Glu-Cys
<b>Dopamin D2 receptor</b>	...Ile-Leu-His-Cys
<b>Dopamin D3 receptor</b>	...Ile-Leu-Ser-Cys
<b>Rat TOM-L1</b>	...His-Ser-Glu-Cys
<b>CD93</b>	...Gly-Thr-Asp-Cys
<b><i>HBc</i></b>	<i>...Glu-Ser-Gln-Cys</i>
<b><i>HBc mod</i></b>	<i>...Glu-Leu-Gln-Cys</i>

\* X, any amino acid, Ψ, hydrophobic. Viral proteins are in *italic*.

What actual role in the life cycle of HBV might play discovered ability of the core protein to interact with GIPC1 remains to be determined. Accumulating data assign to GIPC1 a role of adaptor protein, which couples other proteins to myosin VI movement and participates in recycling of membrane receptors [35]. Since cytoplasmic diffusion operates only within very small volumes, active membrane traffic or cytosolic transport of viral proteins and genome–protein complexes is required. It was determined that HBV capsids are actively transported towards the nucleus with the aid of the cellular microtubule transport system [39]. Whereas the large gap between the cell periphery and the nucleus is usually bridged by the microtubule transport, in the cell periphery and possibly in the nucleus the transport is mediated by the actin system [40]. Thus, one possible function of the interaction between HBc and GIPC1 might be the short-distance intracellular transport of core proteins or capsids.



**Figure 4.** GST pull-down experiment. GST (negative control) and GST fusion proteins were bound to the same amount of glutathione-agarose beads and allowed to interact with HA-tagged proteins. Samples were washed and analyzed in Western blot with anti-GST (top) and anti-HA (bottom) antibodies. Lane 1: GST + HA-HBc; 2: GST + HA-GIPC1; 3: Protein Molecular Weight Marker; 4: GST-GIPC1(124–228) + HA-HBc; 5: GST-GIPC1(124–333) + HA-HBc; 6: GST-HBc(141–183) + HA-GIPC1; 7: GST-HBc + HA-GIPC1.

GIPC1 interacts at least with two other viral proteins–Tax of T-cell leukemia virus type 1 (HTLV1) [44] and E6 of human papillomavirus type 18 (HPV-18) [16], but exact functions of these interactions are unknown. It is known that GIPC1-interacting proteins GLUT1 and NRP1 are involved in HTLV1 entry [17]. Deletion of the Tax PBM in HTLV-1 rendered the mutant virus unable to establish persistent infections in rabbits. It was also noticed that although HTLV-1 and HTLV-2 are close relatives, only HTLV-1 is firmly associated with adult T-cell leukemia or inflammatory neurologic diseases. And only Tax1 protein, but not Tax2, contains a PDZ binding motif at its C-terminus [56]. Similarly, the E6 oncoproteins from high-risk HPV strains contain a PDZ binding motif, whereas those from low-risk isolates do not. Therefore, it is possible that HBc interactions with PDZ-containing proteins might be important in HBV pathogenicity or entry.

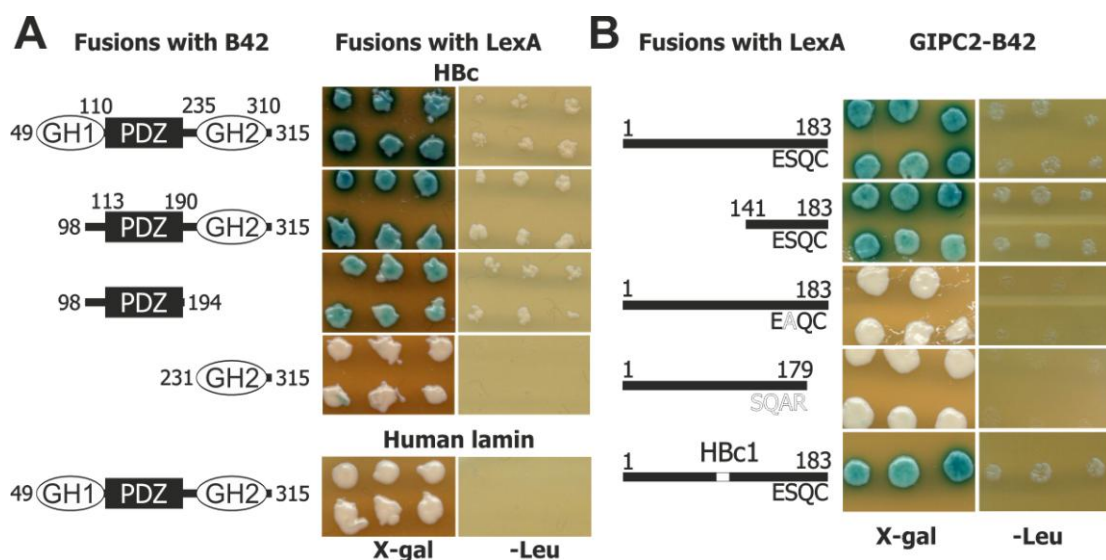
## GIPC2

Another protein specifically interacting with all analyzed HBc proteins was human GIPC2. This protein and its functions are still poorly described, and the name was given because of sequence and domain organization similarity to GIPC1 (which is better known as GIPC). GIPC2 consists of 315 aa and was described bioinformatically approximately at the same time when it was selected in experiments presented here [28]. The third similar protein, GIPC3, was also described bioinformatically [46]. The sequence of GIPC2 protein is 62.0% identical to GIPC1 and 55.3% identical to GIPC3; GIPC1 is 59.9% identical to GIPC3. GIPC2 gene, like GIPC1, consists of six exons, and overall exon-intron structure is very similar in both genes. Expression of GIPC2 is highest in colon, somewhat weaker in kidney and pancreas, also significant in liver. The domain structure of all GIPC proteins is the same: PDZ domain in the central part, N- and C-ends contain newly identified domains of unknown function GH1 and GH2 (GIPC homology domains) [26].

GIPC2 cDNA selected in two-hybrid screen lacks sequence coding the first 48 aa, but all identified domains are intact. Because the function of PDZ domain is to bind other proteins, and we already identified interaction between HBc and PDZ domain of GIPC1,

it was logical to assume that interaction between HBc and GIPC2 was also determined by PDZ domain. To prove this assumption, two-hybrid plasmids containing PDZ and GH2 domains (98-315 aa), PDZ domain only (98-194 aa), and GH2 domain only (231-315 aa) were constructed. Experiments confirmed that PDZ domain alone is sufficient for the interaction with HBc, although protein containing 98-256 aa interacted slightly stronger (**Fig. 5, A**).

Experiments to determine which part of the HBc protein interacts with GIPC2 were also driven by the GIPC2 domain organization similarity to GIPC1. Since PDZ domains usually interact with the C-ends of other proteins, and we already determined that GIPC1 interacts with the C-end of HBc, plasmids available from previous experiments were used to determine interacting region of HBc protein. Experiments revealed that C-end of HBc (141-183 aa) is sufficient for the interaction with GIPC2, whereas HBc with truncated (1-179 aa) or altered (Ser-Gln-Ala-Arg-COOH) C-end was unable to interact. HBc protein with alanine instead of serine, contrary to GIPC1, was unable to interact with GIPC2 (**Fig. 5, B**).



**Figure 5. Interaction between GIPC2 and HBV core protein in yeast two-hybrid system. A:** Determination of GIPC2 interacting region. Truncated forms of GIPC2 protein and human lamin C (negative control) probed for interaction with HBc. **B:** Mapping of HBc interacting region. Nearly full-length GIPC2 (49–315 aa) was probed for interaction with various modifications of HBc and mutant HBc1.

Protein interaction databases contain entries about GIPC2 interactions with several human proteins. Frizzled-3, a protein involved in differentiation and oncogenic transformation, interacts with all three GIPC proteins [26]. The C-end of Frizzled-3 protein contains typical I-class PBM motive, therefore all GIPC proteins are probably able to interact with sequences of this class. However, other GIPC2 interacting proteins: pleiotropin, UNC119, SETDB1, and IGSF21 contain no identifiable PBM motives. GIPC2 protein interacted differently than GIPC1 also with one modification of HBc protein. Therefore, GIPC2 PDZ domain specificity is obviously different from the one of GIPC1, but the range of this specificity remains to be determined.

GIPC2 protein obviously has important role in cellular oncogenic transformation processes. Expression of GIPC2 mRNA is inhibited in cancers of kidney and colon, and activated in some stomach cancers [29]; in cells of acute lymphocytic leukemia this gene

is methylated [30]. However, how this protein participates in processes, what its overall function in the cell, and how its interaction with HBc influences the replication cycle and pathogenesis of HBV, remains to be determined.

There is another question concerning availability of HBc C-end for interaction. Wild-type core proteins usually form homodimers and self-assemble into capsids. To determine whether fusion proteins in two-hybrid experiments are forming similar particles, yeast lysates with expressed HBc fusion proteins were subjected to electron microscopy, but core-like particles were undetected. It is likely that additional domains or low concentration of fusion proteins interfered with particle formation. Although it is generally accepted that arginine-rich C-terminal region of HBc interacts with the viral genome inside the particle, and we did not detect core-like particle formation by fusion proteins, it is still possible that GIPC proteins might interact with intact HBV capsids. There are some data suggesting that at least a fraction of C-termini appear on the external surface of the particle. For example, the arginine-rich protamine-like region of the core protein is responsible for the attachment of nucleocapsids to cell-surface-expressed heparan sulfate [52]; monoclonal antibody directed against this region binds intact HBV capsids; trypsin can clip off this domain from recombinant HBV capsids [8]. It was suggested that peptide at the boundary between the assembly and arginine-rich domains of the core protein forms a mobile array and may allow an extreme mobility of the C-terminal domain [54].

## **Human proteins, interacting only with mutant HBc proteins**

### **Protein with unknown function FLJ20850**

NCBI BLAST alignment of another selected cDNA sequence identified it as product of *Homo sapiens* chromosome 19 open reading frame 60 (C19orf60). The largest cloned cDNA contained coding sequence consisting of 516 nucleotides. It lacks 24 base pairs at the 5'-end according to homologous sequences (for example, [BC012078.1](#)) in nucleic acids databases. Many homologous human sequences in GenBank are products of incorrect splicing with wrong open reading frames. Most human and chimpanzee cDNA sequences have additional 66 nucleotides near the 5'-end. After analysis of *Homo sapiens* chromosome 19 genomic contig (GenBank accession no. [NT\\_011295](#)), it appeared that these cDNAs differ in exon 3 lengths. Some splice site prediction programs find two acceptor sites for intron 2, and popular gene structure prediction server GENSCAN predicts the shorter form of the coding sequence (identified in this screening) (**Fig. 6**). The shorter splicing variant is the only sequenced form in most other species.

The product encoded by this open reading frame is designated as hypothetical protein FLJ20850. The long form of the protein consists of 201 aa, whereas the short form is 179 aa in length. Very similar sequences were found in other mammalian species. Chimpanzee counterpart is 98% identical to human sequence; dog, cattle, and horse sequences have more than 80% identity; rat and mouse sequences share more than 70% of identical amino acids with the human counterpart. More divergent are chicken and zebrafish sequences, which have more than 40% of identical residues (**Fig. 7**). Although this protein is well conserved in mammalian species, search for protein domains and structural motifs in PFAM, SMART and other databases revealed no

significant homology with any known protein domain or structural motif. The class, function, biological process, localization, and site of expression of this protein are undefined in Human Protein Reference Database. However, expression of this mRNA in different human tissues can be evaluated by analyzing EST and microarray databases (**Table 5**). The analysis revealed that FLJ20850 mRNA is expressed in a wide range of human tissues. Interaction of FLJ20850 protein with the MyoD family inhibitor protein was identified by the yeast two-hybrid system (data from BioGRID protein interaction database). This gene was also identified as a site of HBV integration in hepatitis B positive hepatocellular carcinoma cells [33].

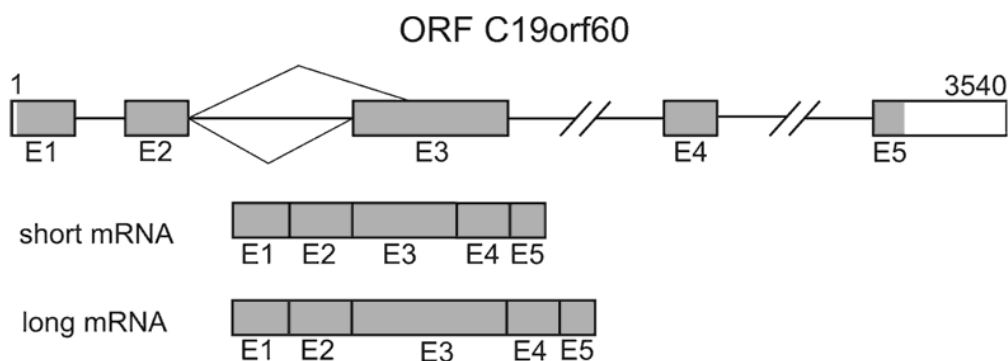
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Exon 1 ▲ Exon 2
E W L A P V Q G L R A W G R G L R V P T C R R G H R Q Y L R
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▲ Exon 3 Intron 3 ▲ Exon 3
S G P D Y D F A R Y R S T V H G V T Q A F A A A S R E V L A
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Exon 3
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acggtggccctgctgcagttgatggagacgcagagctggcggggcaggaggacgctgtacggatgcagcagctgaaaatgaaggttaatt 540
▲ Exon 4 Exon 4 ▲
K T M E A I S E V L Q D L R F D A E S A E *
aaaaccatggaggcgatcagcagaggttctccaggaccttaggtttgatgcggaatctgcggagtga 606
Exon 5

```



**Figure 6. Nucleotide, predicted amino acid sequence and gene structure of FLJ20850.** Fragments missing in the cloned sequence are shadowed. Triangles indicate the boundaries of exons. The point of alternative splicing is grayed out.

Bioinformatic analysis of the FLJ20850 protein revealed no known protein domains giving no clues about possible interacting regions. The lack of significant similarity with other proteins also makes impossible to model 3D structure of this protein. Since the analysis of protein secondary structure predicted three possible coiled-coil regions, we tested which of these structural motifs are indispensable for interaction with HBV core deletion mutants. Truncated forms of the FLJ20850 protein containing first and second, second only, second and third coiled-coil regions were constructed and probed for interaction with HBc1 protein. The experiments revealed that none of the truncated forms were able to interact (**Fig. 4, B**), suggesting that all coiled-coil regions are essential for the interaction with mutated HBc.



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Hsa  MIT-ETAAEPTVFAVPAEEATEARGREEPAWPWKDAPIRTLVQRIHQLOAERAQGFRRLE 60
Ptr  MIT-ETAAERTVFAVPAEEATEARGREEPAWPWKDAPIRTLVQRIHQLOAERAQGFRRLE 60
Eca  MIAAEAAAQSALFAVPGAAAAGVSEREEPAWPWKDAPIRALVQRIHQLOAERAQAFRRLE 61
Bta  MITAEAAESTVFAVPGDTAATGVPEHEELVWPWKDAPIRELVQRIHQLOAERAQAFRRLE 61
Cfa  MITSKAVEDSAVPSVPGTAGAAGVPEREPAWPWKDAPIRALVQRIHQLOAERAQAFRRLE 61
Mmu  MITAAT-E-----VLAADGPGSAEASAWAWRDAPITLVQRIQQLONERAQAFRRLD 51
Rno  MITAAT-E-----VLAADGPGSAEFTWAWRDAPITLVQRIQQLONERAQAFRRLD 51
Gga  MDLWRRCRVA-PCSAP-AGPARSAPYREKPC--HTDEALRALLORFYALOGERVETVRLFE 57
Dre  M-----AP-----SDFKSLVRRRFYVLOSERVEAKKLE 28
Hsa  EGHRYQLRSGPDYDFARYRSTVHGVTQAFAAASREVLAVEAELGGPRRQPLLASHVRSLOE 121
Ptr  EGHRYQLRSPDYDFARYRSTVHEVTQAFAAASREVLAVEAELGGPRRQPLLASHVRSLOE 121
Eca  EGHRYQLHSGPPYDFPRYRSTVHEVTQAFAAASREVLAVEAELAGPRAQPLLASHVRSLOQ 122
Bta  EGHRYQLSSGPPYDFPRYRSTVHEVTQVFAAASREVLAVEAELAGPRAQPLLASHVRSLOQ 122
Cfa  EGHRYQLRSGPPYDFPRYRSTVHEVTQAFAAASREVLAVEAELAGPRAQPLLASHVRSLOQ 122
Mmu  QAHRYQLLSGQHYDFPSYRSVVEVTQAFAAASREVLAVEAELAGPRAQPVLARHVRSLOE 112
Rno  QAHRYQLLSDLHDFPSYRSVVEVTQAFAAASREVLAVEAELAGPRAQPVLARHVRSLOE 112
Gga  EGHRYQLSSAPHYDFPRYRSTVHEVTAAFSGISREVLQIQRLRGELGRPDLAQHIIRLQE 118
Dre  EGHEAYLRTGFHYDFEYHYKQIVHEITKAFCGISKEVLTIKQQLHQDFDRPDLSEHIDKLOI 89
Hsa  LEQTRLGTVALQLMTEPELAGQEQE---DAVRMQLKMKVIKTMEAISEVLQDLRFDAESAE 179
Ptr  LEQTRLGTVALQLMTEPELAGQEQE---DAVRMQLKMKVIKTMEAISEVLQDLRFDAESAE 179
Eca  LEETRLATVALLQMGAPELTEQEQE---DPLQMHQLKMKVIKTMEAISEVLQDLRFDAESSE 180
Bta  LEETRLITVALLQMGTEPELIGQEQE---DSLQMHQLKMKVIKTMEAISEVLQDLRFDAESAE 180
Cfa  LEQTRLATVALLQMGAPELTEQEQE---NTLEMHQLKMKVIKTMEAISEVLQDLRFDAESAE 180
Mmu  LEQTRLATVALLOVMGTEGVSEQEQE---DPEKLHQLKMKVIKTMEAISEVLQDLRFDAESAE 169
Rno  LEQTRLATVALLOVMGTEGVSEQEQE---DPEKLHQLKMKVIKTMEAISEVLQDLRFDAESAE 169
Gga  REQEKIQLTAQLQLARQRAQDEPSVDTHQEQEVRELKH---RSDAVLSACYRGCRLFSICHF 176
Dre  KEKEKLELTAKLQLTRQSAQDHPEDDFQEKVREIKQEI IKNTAALSEILQDFKFDSEPE 150

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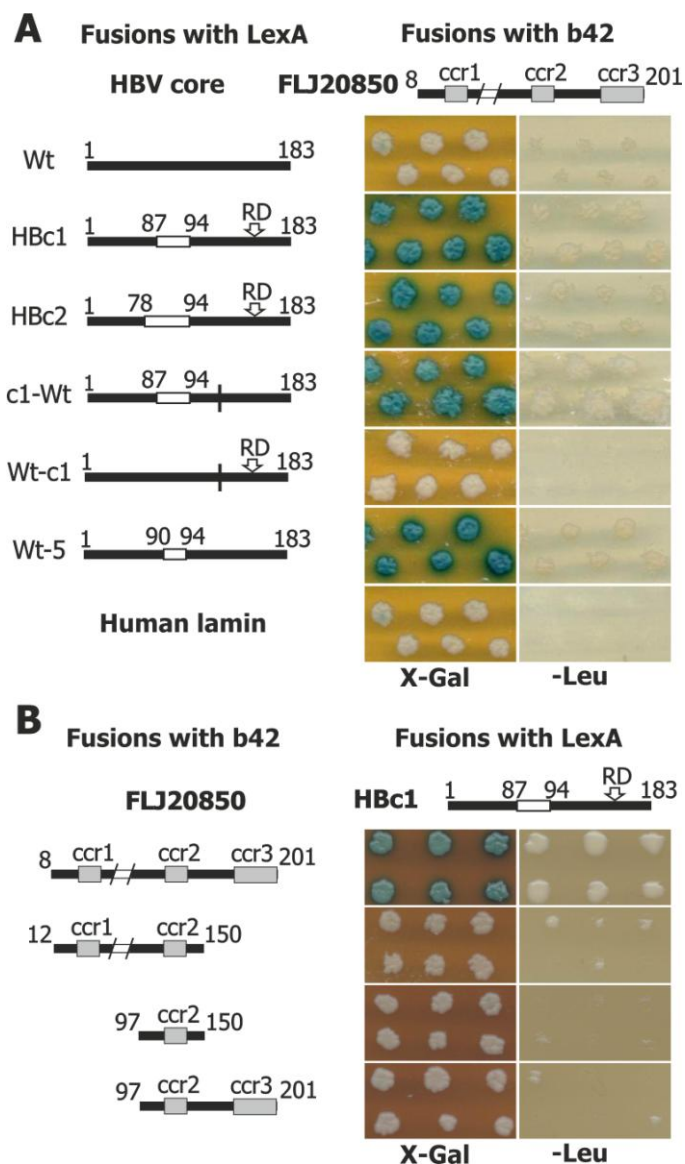
**Figure 7. Multiple sequence alignment of human protein FLJ20850 and its homologs from other organisms.** Species identifiers: Hsa, *Homo sapiens*; Ptr, *Pan troglodytes*; Eca, *Equus caballus*; Bta, *Bos taurus*; Cfa, *Canis lupus familiaris*; Mmu, *Mus musculus*; Rno, *Rattus norvegicus*; Gga, *Gallus gallus*; Dre, *Danio rerio*. *Homo sapiens* and *Pan troglodytes* sequences are shorter splicing variants. *Gallus gallus* sequence is truncated.

**Table 5. Expression of FLJ20850 mRNA in various human tissues**

Tissue	EST profile (tpm)	Microarray expression data		
		Gene Atlas	GeneNote	BOE
kidney	98	+	+	+
prostate	94	++	++	++
ovary	87	+	ND	+
spleen	73	ND	++	++
heart	66	++	++	+
pancreas	60	+	+	+
adrenal	60	+	ND	+
liver	38	++	+	+
brain	34	++	++	++
lung	32	++	++	++
placenta	31	+	ND	+
intestine	25	ND	ND	++
thymus	24	+	++	++
muscle	9	++	++	++
testis	3	+	ND	+

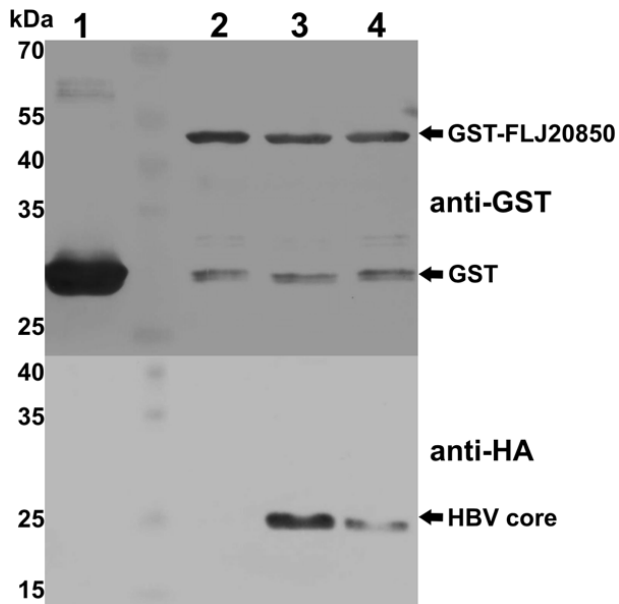
EST profile: approximate gene expression patterns as inferred from EST counts and cDNA library sources represented in transcripts per million (data from NCBI). *Gene Atlas*: Novartis Gene Expression Atlas project; data from *GEO* GDS596 and *ArrayExpress* E-AFMX-5 datasets. *GeneNote*: Gene Normal Tissue Expression project; data from *GEO* GDS423 dataset. *BOE*: genome-wide survey of breadth of expression in normal tissues; data from *GEO* GDS1096 and *ArrayExpress* E-GEOD-2361 datasets. ++: expression above average; +: expression below average; ND – no data.

It is unclear also which region of the mutated core proteins is responsible for the interaction with the FLJ20850 protein. This interaction could occur in the area with deletions, where local protein structure is altered. But there is also a possibility that deletions make some other protein area available for the interaction. In addition, the deletion mutants, which belong to the genotype A, differ from the genotype D wt protein by additional arginine and asparagine insertion after amino acid 151. To test which part of the protein takes part in the interaction, hybrid proteins containing the N-part of the wt protein fused to the C-part of the HBc1 mutant (wt-c1) and the N-part of the HBc1 protein fused to the C-part of the wt protein (c1-wt) were constructed and probed for interaction with FLJ20850 protein. The results indicate that the protein part with deletion is determining the interaction, whereas two additional amino acids in the C-part of the core protein do not contribute to the interaction (**Fig. 8, A**). To proof that introduced deletion confers ability to interact, wt protein with deletion 89-93 was constructed, and it was able to interact with FLJ20850 (Fig. 4A). The results of two-hybrid experiments imply that deletions in the central part of the core protein confers the ability to interact with the FLJ20850 protein, although it is still unclear which amino acids of both proteins participate in the interaction.



**Figure 4.** Interaction between HBV core deletion mutants and FLJ20850 protein. **A:** An attempt to define interacting regions of HBV core mutants. *Wt*, wild-type HBV core; *HBc1* and *HBc2*, HBV core deletion mutants; *c1-Wt*, hybrid core protein with N-part of HBc1 mutant and C-part of wt protein (vertical bar represents junction site); *Wt-c1*, hybrid core protein with N-part from wt and C-part from HBc1 mutant; *Wt-5*, wt core with five amino acids deletion in the central region. *RD*, two additional amino acids, absent in wt protein. White bar represents deletion area. **B:** An attempt to define interaction region of the FLJ20850 protein. White bar represents a stretch of amino acids present in longer form of protein; *ccr*, theoretical coiled-coil regions.

Interaction between core mutants and the FLJ20850 protein was tested also by GST pull-down assay. Expression of FLJ20850 fusions with GST and HA tags in *E.coli* resulted in clearly discernable bands of expected size. Nonetheless, substantially smaller quantities of GST-FLJ20850 were able to bind to glutathione-beads than samples with GST alone, which can be explained by partial degradation of the fusion protein. Assay confirmed that both core deletion mutants, but not wt core, are able to interact with GST-FLJ20850 protein (**Fig. 9**).



**Figure 9.** Interaction between HBV core deletion mutants and FLJ20850 protein in GST pull-down assay. GST (negative control) and GST fusion proteins were bound to the same amount of glutathione-agarose beads and allowed to interact with HA-tagged proteins. Samples were washed and analyzed in Western blot with anti-GST (top) and anti-HA (bottom) antibodies. Lane 1: GST + Hbc1; lane 2: GST-FLJ20850 + wt; lane 3: GST-FLJ20850 + Hbc1; lane 4: GST-FLJ20850 + Hbc2. *Wt*, wild-type HBV core protein; *Hbc1*, core variant with deletion 86-93; *Hbc2*, core variant with deletion 77-93.

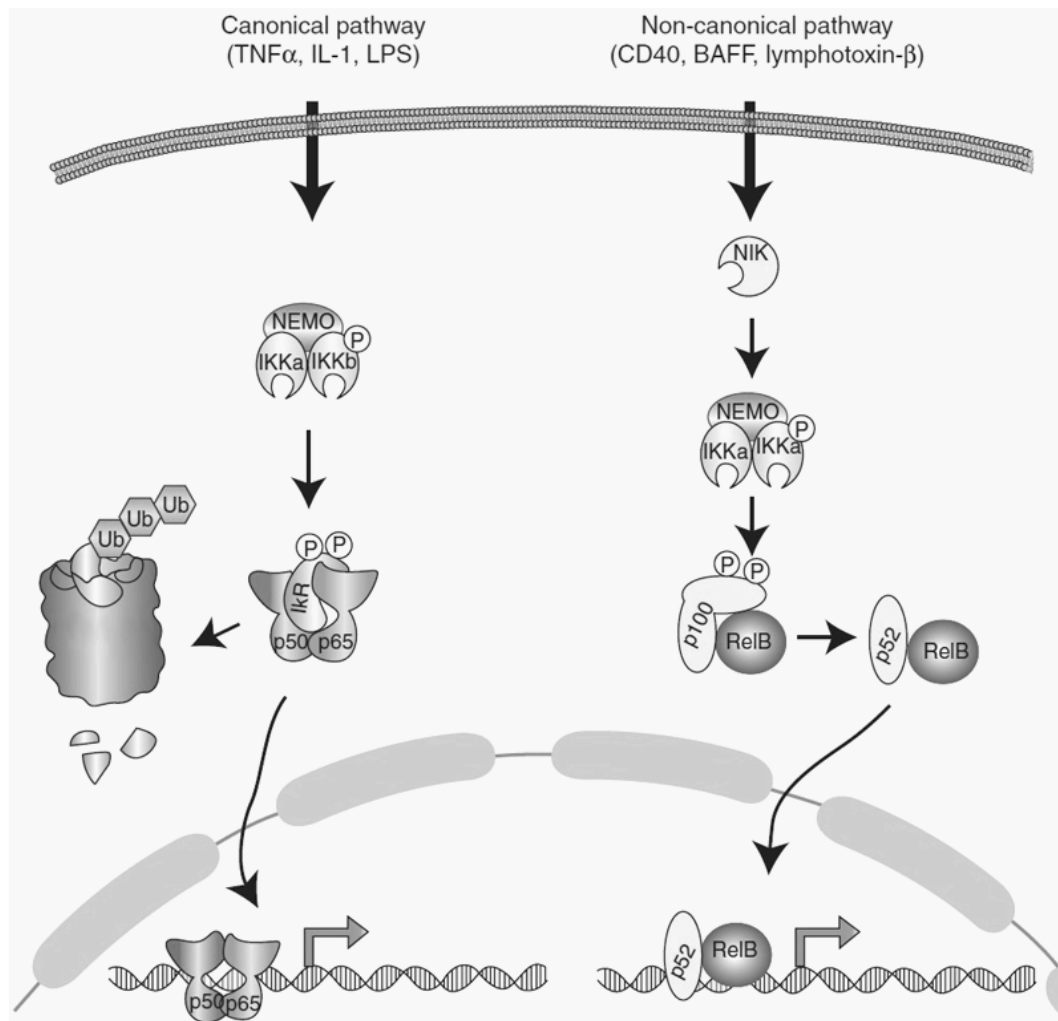
In summary, human protein FLJ20850, which is well conserved among mammalian species but has no assigned function, interacted specifically with Hbc deletion variants, but not with the WT protein. It remains to determine the cellular function of the FLJ20850 protein and the role of the identified interaction in enhanced pathogenesis of mutant viruses.

### IKK $\gamma$ (NEMO)

Another protein specifically interacting only with Hbc mutants was IKK $\gamma$  (**gamma** subunit of **I** $\kappa$ **B** **k**inase complex), also named NEMO (**NF**- $\kappa$ **B** **e**ssential **m**odulator). This protein is essential for formation of IKK complex, regulator of transcription factor NF- $\kappa$ B activity [23, 43]. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. Inducible NF- $\kappa$ B activation depends on phosphorylation-induced proteosomal degradation of the inhibitor of NF- $\kappa$ B proteins (I $\kappa$ Bs), which retain inactive NF- $\kappa$ B dimers in the cytosol of unstimulated cells. The majority of the diverse signaling pathways that lead to NF- $\kappa$ B activation converge on the I $\kappa$ B kinase (IKK) complex composed of three proteins (IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ ), which is responsible for I $\kappa$ B phosphorylation and is essential for signal transduction to NF- $\kappa$ B [37].

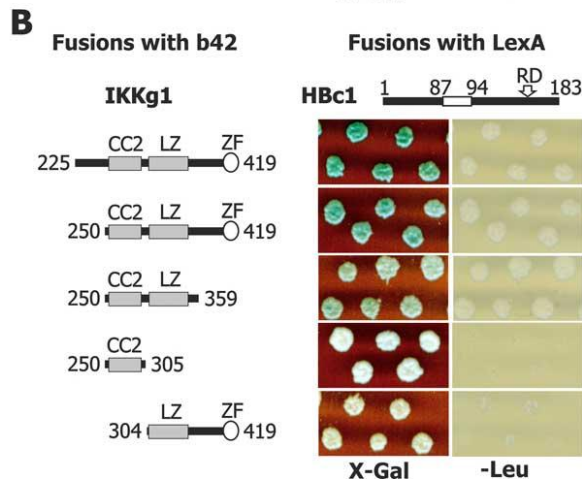
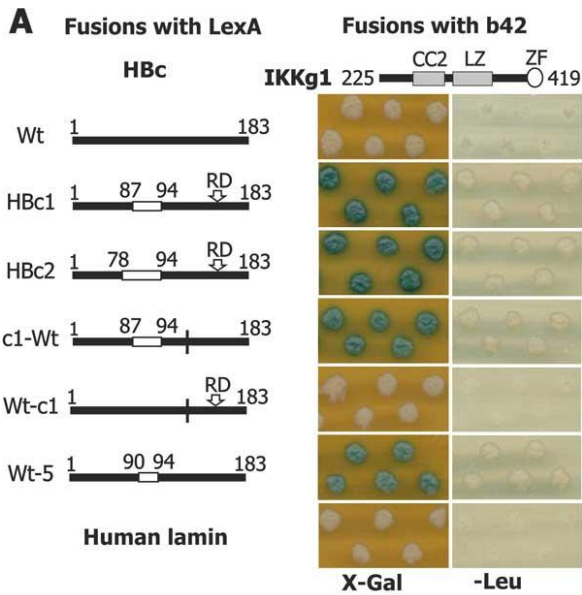
Experiments with IKK $\alpha$  and IKK $\beta$  knockout mice revealed that IKK $\beta$  subunit more is important in regulating the canonical NF- $\kappa$ B activation pathway, whereas IKK $\alpha$  is essential in regulating the alternative pathway (**Fig. 10**). IKK $\gamma$  is obligatory for canonical pathway, whereas alternative pathway depends on activation by kinase NIK. However,

new data suggest that both pathways are not strictly separated, and all kinases participate in regulating both activation pathways, although at different extent.

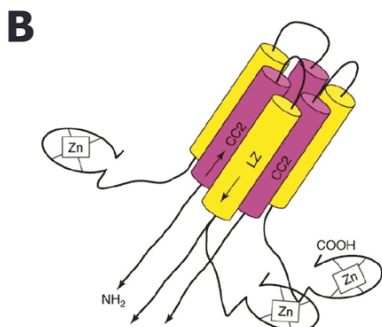
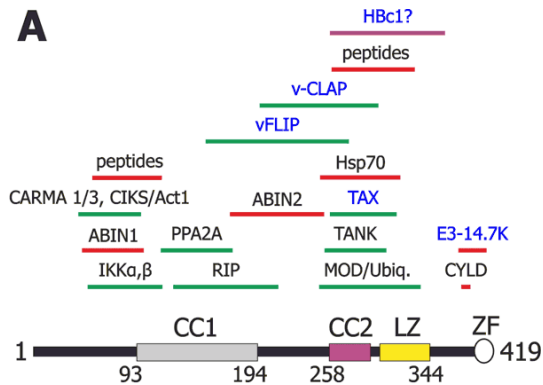


**Figure 10. Canonical and non-canonical signaling to NF- $\kappa$ B.** The canonical pathway is induced by TNF $\alpha$ , IL-1, or LPS and uses a large variety of signaling adaptors to engage IKK activity. Phosphorylation of serine residues in the signal responsive region (SRR) of classical I $\kappa$ Bs by IKK $\beta$  leads to I $\kappa$ B ubiquitination and subsequent proteosomal degradation. This results in release of the NF- $\kappa$ B dimer, which can then translocate to the nucleus and induce transcription of target genes. The alternative pathway depends on NIK (NF $\kappa$ B-inducing kinase) induced activation of IKK $\alpha$ . IKK $\alpha$  phosphorylates the p100 NF- $\kappa$ B subunit, which leads to proteosomal processing of p100 to p52. This results in the activation of p52-RelB dimers, which target specific  $\kappa$ B elements. According to Oeckinghaus and Ghosh [37].

An attempt to determine interacting regions of mutated HBC protein was preceded with similar considerations that are already described in the section about FLJ20850 protein. It was needed to take into account not only the area with deletions, but also other possibly exposed areas as well as genotype A of the mutated protein. Therefore, HBC protein modifications made to test interaction with FLJ20850 protein were also used to test interaction with IKK $\gamma$ . Experiments in the yeast two-hybrid system revealed that the C-part of the core protein do not contribute to the interaction, and deletion of five amino acids introduced into the central region of the HBC protein was sufficient to induce interaction between IKK $\gamma$  and modified HBC (**Fig. 11, A**).



**Fig. 11. Interaction of IKKy protein with HBc mutants.** **A:** An attempt to define interacting regions of HBV core mutants. *Wt*, wild-type HBV core; *HBc1* and *HBc2*, HBV core deletion mutants; *c1-Wt*, hybrid core protein with N-part of *HBc1* mutant and C-part of *wt* protein (vertical bar represents junction site); *Wt-c1*, hybrid core protein with N-part from *wt* and C-part from *HBc1* mutant; *Wt-5*, *wt* core with five amino acids deletion in the central region. *RD*, two additional amino acids, absent in *wt* protein. White bar represents deletion area. **B:** An attempt to define interaction region of the IKKy protein. *CC2*, second coil-coiled region; *LZ*, leucine zipper domain; *ZF*, zinc finger domain.



**Fig. 12. IKKy protein.** **A:** The domain structure of IKKy protein and regions interacting with other proteins. *CC1* and *CC2*, coil-coiled regions; *LZ*, leucine zipper domain, *ZF*, zinc finger domain. Green bars mark interacting regions of activating proteins, red bars mark regions of inhibiting proteins. Names of viral proteins are in blue. **B:** The scheme of the C-end of active IKKy trimer.

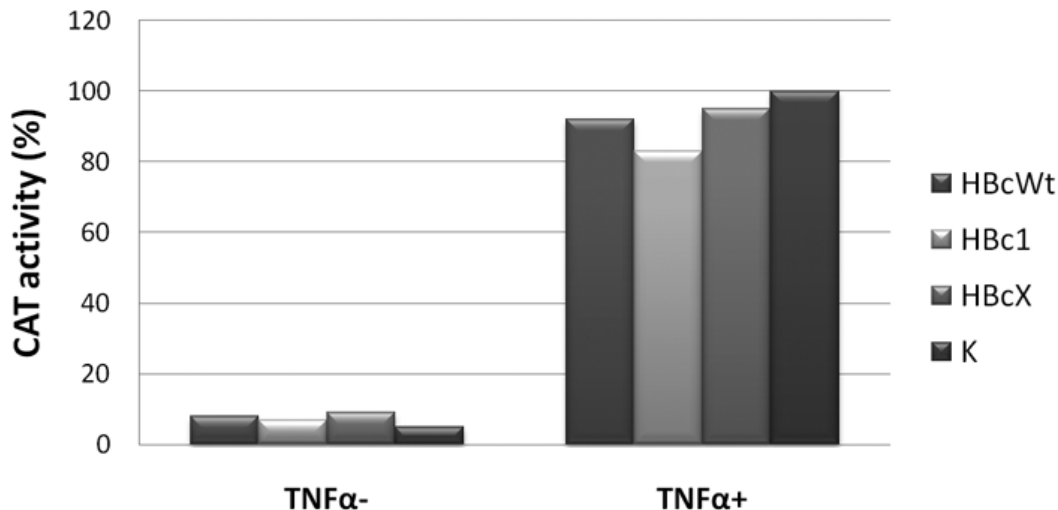
IKK $\gamma$  is very conservative protein of 419 aa, consisting of several domains and other functionally important regions. Human cDNA cloned in this work contains 195 C-end amino acids, spanning second coil-coiled region, leucine zipper domain, and zinc finger domain. To determine which regions of IKK $\gamma$  protein are important for interaction with mutant HBc proteins, two-hybrid plasmids containing all three domains (250-419 aa), second coil-coiled region and leucine zipper domain (250-359 aa), leucine zipper domain and zinc finger domain (304-419 aa), and second coil-coiled region only (250-305 aa) were constructed. Experiments revealed that this interaction requires the second coil-coiled region and leucine zipper domain of IKK $\gamma$ , although the entire C-end after 250 aa interacts slightly stronger (**Fig. 11, B**).

Although exact mechanism of IKK $\gamma$  activation is unclear, available data suggests that this activation needs oligomerization, ubiquitination, and phosphorylation of IKK $\gamma$ . IKK $\gamma$  oligomerization is determined by two regions: minimal oligomerization domain (MOD, spans 250–360 aa) and N-end region, usually interacting with other members of IKK complex. Peptides, preventing IKK $\gamma$  interaction with other IKK kinases or C-end driven oligomerization, prevent NF- $\kappa$ B activation by external stimuli. IKK $\gamma$  protein and other NF- $\kappa$ B pathway components are popular targets of viral immune-modulating proteins. HTLV-1 Tax protein, Kaposi's sarcoma associated herpesvirus vFLIP protein, and vCLAP of equine herpesvirus-2 activate IKK complex and NF- $\kappa$ B by inducing IKK $\gamma$  oligomerization. E3-14.7K protein of adenovirus-2 and vaccinia virus B14 protein inhibit IKK complex by interacting with IKK $\gamma$ . Available data indicates that various proteins, interacting with different regions of IKK $\gamma$ , can activate IKK or prevent it from activation (**Fig. 12**). The second coil-coiled region and leucine zipper domain of IKK $\gamma$  also interacts with activating as well as with inhibiting proteins; therefore, the role of IKK $\gamma$  interaction with mutant HBc proteins needs to be determined experimentally.

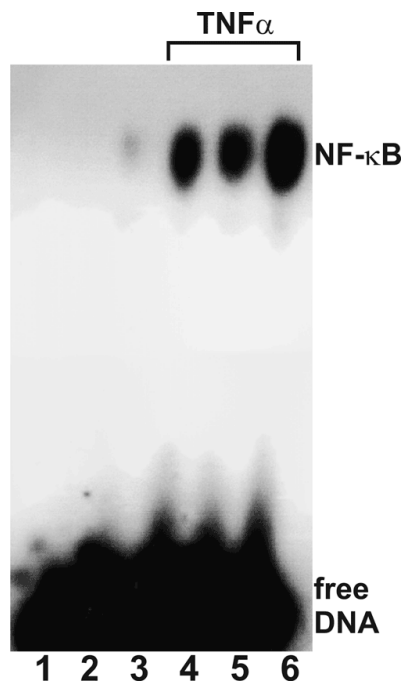
The first experiment employed granulocyte-monocyte progenitor cell line HL-60 and plasmid pRR55, which contains HCMV IE enhancer/promoter region upstream of CAT reporter gene. HCMV IE promoter activity in HL-60 cells is regulated by NF- $\kappa$ B. The cells were transfected with plasmids for expression of HBc wt gene (pRL-CMV-HBc), mutant HBc1 (pRL-CMV-HBc1), and control plasmid with reversed HBc wt gene (pRL-CMV-HBcX). Half of the transfected cells were treated with TNF- $\alpha$ , which induces canonical NF- $\kappa$ B activation pathway. Measured CAT activity was supposed to indicate the level of NF- $\kappa$ B activation (**Fig. 13**). The second experiment was electrophoretic mobility shift assay (EMSA). Human hepatocyte cell line Huh7 was transfected with plasmids pRL-CMV-HBc and pRL-CMV-HBc1. Before disruption, half of cells were treated with TNF- $\alpha$ , and protein extracts from isolated nuclei were mixed with radioactively labeled control oligonucleotides and oligonucleotides containing NF- $\kappa$ B binding site. Mobility shift of specific oligonucleotides was indicative for bound active NF- $\kappa$ B (**Fig. 14**).

Both experiments showed no activation of NF- $\kappa$ B and no distinct inhibition of NF- $\kappa$ B activation by TNF- $\alpha$ . These experimental results indicate that mutant HBc1 is not capable to activate cellular NF- $\kappa$ B, but whether it can prevent NF- $\kappa$ B activation by TNF- $\alpha$  is still an open question. Although expression of HBc proteins in human cells was driven by powerful HCMV IE promoter, the level of the synthesis was low. Moreover, HCMV IE promoter itself contains NF- $\kappa$ B binding sites, so if HBc1 prevents NF- $\kappa$ B activation, it can also inhibit its own synthesis. There is also a possibility that TNF- $\alpha$  can activate NF- $\kappa$ B

without involvement of IKK $\gamma$ , because recent investigation revealed that TNF- $\alpha$  activates IKK complex by two independent ways: intermediate signaling proteins MAP3K phosphorylate IKK $\beta$ , and another intermediate protein TRAF2 induces ubiquitilation of IKK $\gamma$  [31]. Therefore, it would be helpful to investigate the interference of mutant HBc proteins with NF- $\kappa$ B activation by other signals and to make experiments with other cell lines and promoters for expression of HBc proteins.



**Fig. 13. CAT assay to measure how activity of NF- $\kappa$ B regulated promoter is modulated by HBc variants.** Human cell line HL-60 transfected with plasmids pRR55 (containing CAT gene downstream the NF- $\kappa$ B regulated HCMV promoter) and pRL-CMV-HBcWt, pRL-CMV-HBc1, or pRL-CMV-HBcX, which contain expression cassettes for HBc wt, mutant HBc1 and inverted HBc wt genes respectively. *K*, control cells transfected only with pRR55. Half of cells from each group were treated with TNF- $\alpha$ . CAT activity is presented as percentage of activity in cells transfected only with pRR55 and treated with TNF- $\alpha$ .



**Fig. 14. EMSA assay to measure how HBc variants modulate NF- $\kappa$ B activity.** Human hepatocyte cell line Huh7 was transfected with plasmids pRL-CMV-HBc (lanes 1 and 4) and pRL-CMV-HBc1 (lanes 2 and 5), in which HCMV IE promoter drives expression of HBc wt and mutant HBc1 respectively. Lanes 3 and 6 contains samples from untransfected Huh7 cells. One group of cells (4-6 lanes) were treated with TNF- $\alpha$  two hours before disruption. Oligonucleotides bound with activated NF- $\kappa$ B are on the top, unbound oligonucleotides are at the bottom.

Although still unclear how NF- $\kappa$ B activity or other cellular processes are modulated by HBc1 interaction with IKK $\gamma$ , it is likely that this interaction might be important in liver damage caused by mutated HBV. Transgenic IKK $\gamma$  knockout mice [45] or mice with

hepatocyte-specific IKK $\gamma$  deletion [4] suffer from severe liver damage and underdevelopment. Although inactive in normal liver cells, transcription factor NF- $\kappa$ B is permanently upregulated in hepatocarcinoma cells and in liver damaged by various types of hepatitis [34]. Its activity is also regulated by hepatitis B and C viruses, and especially by HCV core protein. In hepatitis B infected hepatocytes NF- $\kappa$ B is activated by HBV X protein to activate NF- $\kappa$ B-regulated HBV promoters [10]. It is already known that inhibition of NF- $\kappa$ B activity stops replication of cancerous cells, but inhibition of NF- $\kappa$ B activity and simultaneous stimulation with TNF- $\alpha$  leads to cell death by apoptosis. Therefore, inhibition of NF- $\kappa$ B activation by mutant Hbc proteins and simultaneous stimulation by X protein during mutant HBV infection could lead to observed liver damage associated with mutant core proteins.

## Overview of the results

Hepatitis B virus mutants with in-frame deletions in the central part of the core gene are associated with a severe course of infection in long-term immunosuppressed renal transplant recipients. In this study, yeast two-hybrid system was employed to investigate interaction capabilities of core mutants with deletions in the central region. The wild-type core protein was able to interact with the wt as well as with the deletion variants Hbc1 and Hbc2. The wild-type protein also weakly interacted with another two mutants – Hbc5 and Hbc6. These results support the possibility that mutant core variants might contribute to wild-type virus inhibition by forming dimers with the wt Hbc proteins. Most mutant proteins were unable to form homodimers – a prerequisite for the formation of intact core particle. Therefore, this experiment also supports the assumption that the presence of wt HBV virus is required by most mutant viruses to provide viable core particles, necessary for the completion of the virus life cycle.

To find host proteins possibly involved in enhanced pathogenesis of mutant HBV variants, a human hepatocyte cDNA library was screened for proteins interacting with the mutant, but not with the wt core protein. Human protein FLJ20850, which is well conserved among mammalian species but has no assigned function, interacted specifically with Hbc deletion variants, but not with the wt protein. An attempt to determine interacting regions revealed that FLJ20850 was unable to interact without significant parts of its C- or N-end, and introduced deletion in the central region conferred interaction capability to the wt core protein. It remains to determine the cellular function of the FLJ20850 protein and the role of the identified interaction in enhanced pathogenesis of mutant viruses.

Another protein specifically interacting only with mutant Hbc protein was IKK $\gamma$ . Experiments revealed that this interaction requires second coil-coiled region and leucine zipper domain of IKK $\gamma$ , although entire C-end after 250 aa interacts slightly stronger. IKK $\gamma$  is important regulator of transcription factor NF- $\kappa$ B, associated with liver pathogenesis and actively regulated by various viral proteins. Experiments aimed to determine the influence of Hbc1 expression on NF- $\kappa$ B activity showed no activation of NF- $\kappa$ B and no distinct inhibition of NF- $\kappa$ B activation by TNF- $\alpha$ . Although still unclear how NF- $\kappa$ B activity or other cellular processes are influenced by Hbc1 interaction with IKK $\gamma$ , it is likely that this interaction might be important in liver damage caused by mutated HBV.



Up to now little is known about wild type HBc interactions with host cell proteins, although such interactions might be essential for virus propagation and pathogenicity. In this work, we also identified several human proteins interacting with wild type HBc. Among several selected proteins, human GIPC1 interacted most strongly. Common protein interaction domain PDZ was identified as the region, sufficient for the interaction with HBc. A putative C-terminal PDZ-interacting motif was identified inside the core protein, and this sequence proved to be important for this interaction. Interaction between HBc and GIPC1 might be important for the short-distance intracellular transport of core proteins or capsids, HBV pathogenicity or entry, but the actual role of this interaction in the life cycle of HBV remains to be determined.

Another human protein with the same domain structure and homologous sequence, GIPC2, was also identified as HBc-interacting partner. This interaction was also dependent on GIPC2 PDZ domain and putative PDZ-interacting motif of HBc. GIPC2 PDZ domain specificity appeared to be different from the one of GIPC1, but exact range of this specificity is unknown. Cellular function of this protein as well as importance of its interaction in HBV replication cycle or pathogenesis so far is unknown.

## CONCLUSIONS

1. Human liver cDNA library was screened for proteins interacting with hepatitis B virus by means of yeast two-hybrid system, and proteins GIPC1, GIPC2, RPL5, and fibrinogens alpha and gamma were selected.
2. Human proteins FLJ20850, IKK $\gamma$  (NEMO), KIF13A, NOTCH2NL, fumarase, and CASKIN2 interacted only with highly pathogenic HBV core mutant, but not with the wild type protein.
3. Common protein interaction domain PDZ of GIPC1 interacted with identified putative PDZ interaction motif at the C-end of HBc protein, and amino acid composition of identified sequence proved to be important for his interaction.
4. The PDZ domain of GIPC2 protein was identified to interact with the putative PDZ interaction motif of the HBc protein.
5. Interaction between protein of unknown function FLJ20850 and HBc1 mutant is determined by naturally occurring or artificially induced deletion in the central part of the HBc protein. The gene of FLJ20850 protein was analyzed bioinformatically and its expression evaluated in different human tissues.
6. Interaction between IKK $\gamma$  protein and HBc1 mutant is determined by the second coil-coiled region and leucine zipper domain of IKK $\gamma$  and naturally occurring or artificially introduced deletion in the central part of the HBc protein. Attempts to reveal how detected interaction modulates the activity of transcription factor NF- $\kappa$ B, identified no significant positive or negative regulation.

## LIST OF PUBLICATIONS

### The thesis is based on the following original publications:

1. **Razanskas R**, Sasnauskas K. 2010. Interaction of hepatitis B virus core protein with human GIPC1. *Archives of Virology*. 155(2):247-50.
2. **Razanskas R**, Sasnauskas K. 2009. A novel human protein is able to interact with hepatitis B virus core deletion mutant but not with the wild-type protein. *Virus Research*. 146(1-2):130-4.
3. **Ražanskas R**, Sasnauskas K. 2003. Human proteins interacting with human hepatitis B core antigen. *Biologija*. 1:59-61.

### Other publications:

1. Pumpens P, **Razanskas R**, Pushko P, Renhof R, Gusars I, Skrastina D, Ose V, Borisova G, Sominskaya I, Petrovskis I, Jansons J, Sasnauskas K. 2002. Evaluation of HBs, HBc, and frCP Virus-Like Particles for Expression of Human Papillomavirus 16 E7 Oncoprotein Epitopes. *Intervirology*. 45(1):24-32.
2. **Ražanskas R**, Pumpens P, Pushko P, Sasnauskas K. 2000. Immunogenicity of chimeric HBsAg-HPV16 E7 viral protein. *Biologija*. 1:42-44.
3. Sasnauskas K, Buzaitė O, Vogel F, Jandrig B, **Razanskas R**, Staniulis J, Scherneck S, Krüger DH and Ulrich R. 1999. Yeast cells allow the high-level expression and formation of polyomavirus - virus like particles. *Biological Chemistry*. 380:381-386.
4. **Razanskas R**. 1997. Construction and expression of hybrid genes carrying HBV HBs and HPV type 16 E7 coding sequences in yeast. *Biologija*. 1:44-46.
5. **Ražanskas R**, Pumpens P, Sasnauskas K. 1996. Construction and expression in yeast hybrid genes carrying HIV1 gp120 V3-loop coding sequence. *Biologija* 2:45-47.

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## CURRICULUM VITAE

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## REZIUMĖ

Šiame darbe buvo tyrinėjami HBV šerdies baltymų ir padidinto patogeniškumo mutantų tarpusavio sąveika bei jų sąveika su žmogaus kepenų baltymais. Naudojant mielių dviejų hibridų sistemą nustatyta, kad dauguma mutantinių HBc baltymų negali sąveikauti tarpusavyje. Šis rezultatas paremia prielaidą, kad pacientų organizmuose šalia mutantinių virusų visada aptinkami laukinio tipo virusai reikalingi dėl pirmųjų nesugebėjimo suformuoti pilnavertes šerdies daleles. Taip pat nustatyta, kad kai kurių mutantinių virusų pakitę HBc baltymai gali sąveikauti su laukinio tipo HBc. Šis rezultatas paremia prielaidą, kad prie šių mutantinių virusų vykdomo laukinio tipo HBV dauginimosi slopinimo gali prisidėti ir pakitusių HBc baltymų sąveika su laukinio tipo HBc, taip galimai suformuojant netaisyklingas, greitai degraduojamas daleles.

Žmogaus kepenų kDNR bibliotekoje dviejų hibridų metodu ieškant baltymų, sąveikaujančių su laukinio tipo arba mutantiniais HBc baltymais, atrinkti dviejų tipų žmogaus baltymai – sąveikaujantys vien su mutantiniais HBc ir sąveikaujantys tiek su mutantais HBc1 bei HBc2, tiek su laukinio tipo HBc baltymais. Su visomis tirtomis HBc atmainomis sąveikavo GIPC1, GIPC2, ribosominis baltymas L5 ir fibrinogenai alfa bei gama. Detaliau tiriant HBc baltymų sąveiką su GIPC1 baltymu nustatyta, kad tarpusavyje sąveikauja HBc baltymo C-galas ir GIPC1 baltymo PDZ domenas. HBc baltymo C-gale aptiktas PDZ domenų atpažįstamos sekos motyvas ir parodyta, kad šios sekos pokyčiai įtakoja HBc sąveiką su GIPC1. HBc ir GIPC1 sąveika patvirtinta GST ištraukimo metodu. Detaliau tiriant HBc ir GIPC2 sąveiką nustatyta, kad šiuo atveju taip pat sąveikauja HBc C-galas ir GIPC2 PDZ domenas, o galinių HBc baltymo amino rūgščių seka įtakoja šių baltymų tarpusavio sąveiką.

Vien su mutantais HBc1 ir HBc2 sąveikavo žmogaus baltymai FLJ20850, IKK $\gamma$  (NEMO), KIF13A, NOTCH2NL, fumarazė ir CASKIN2. Pirmą kartą aprašomo nežinomos funkcijos žmogaus baltymo FLJ20850 raiška ir geno struktūra apibūdinta naudojantis bioinformatinėmis duomenų bazėmis. Detaliau tiriant HBc mutantų ir FLJ20850 sąveiką nustatyta, kad jai vykti reikalinga natūraliai aptinkama arba dirbtinai įvesta HBc centrinės dalies iškrita, o pašalinus pasirinktas FLJ20850 sritis ši sąveika nevyko. Mutanto HBc1 ir FLJ20850 sąveika patvirtinta GST ištraukimo metodu. Detaliau tiriant HBc mutantų ir IKK $\gamma$  sąveiką nustatyta, kad šiai sąveikai vykti reikalinga natūraliai aptinkama arba dirbtinai įvesta HBc centrinės dalies iškrita, o IKK $\gamma$  baltyme šią sąveiką apsprendžia antroji spiralizuotų vijų sritis ir leucino užtrauktuko domenas. Gerai žinoma, kad IKK $\gamma$  baltymo reguliuojamo transkripcijos veiksnio NF- $\kappa$ B aktyvumą įtakoja daugelis virusų, šis transkripcijos reguliatorius labai svarbus ir kepenų patogenezėje. Bet mutanto HBc1 vykdomo NF- $\kappa$ B aktyvavimo ar žymesnio jo slopinimo žmogaus ląstelėse naudotais metodais aptikti nepavyko.

Darbo metu gauti rezultatai nurodo naujas laukinio tipo ir mutantinių HBV sąveikos su šeimininko ląstele tyrimų kryptis ir parodo potencialiai svarbius šiai sąveikai žmogaus baltymus. Detalus šių baltymų reikšmės HBV dauginimosi ciklui ir įvairių viruso atmainų

patogeniškumui ištyrimas, kai kurių aptiktų baltymų funkcijos ląstelėje nustatymas, galėtų praplėsti virusų biologijos ir patogeniškumo supratimą bei padėti kurti naujas antivirusines priemones.

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