The Protein Kinase IKKε Can Inhibit HCV Expression Independently of IFN and Its Own Expression Is Downregulated in HCV-Infected Livers

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During a viral infection, binding of viral double-stranded RNAs (dsRNAs) to the cytosolic RNA helicase RIG-1 leads to recruitment of the mitochondria-associated Cardif protein, involved in activation of the IRF3-phosphorylating IKKε/TBK1 kinases, interferon (IFN) induction, and development of the innate immune response. The hepatitis C virus (HCV) NS3/4A protease cleaves Cardif and abrogates both IKKε/TBK1 activation and IFN induction. By using an HCV replicon model, we previously showed that ectopic overexpression of IKKε can inhibit HCV expression. Here, analysis of the IKKε transcriptome profile in these HCV replicon cells showed induction of several genes associated with the antiviral action of IFN. Interestingly, IKKε still inhibits HCV expression in the presence of neutralizing antibodies to IFN receptors or in the presence of a dominant negative STAT1α mutant. This suggests that good IKKε expression levels are important for rapid activation of the cellular antiviral response in HCV-infected cells, in addition to provoking IFN induction. To determine the physiological importance of IKKε in HCV infection, we then analyzed its expression levels in liver biopsy specimens from HCV-infected patients. This analysis also included genes of the IFN induction pathway (RIG-I, MDA5, LGP2, Cardif, TBK1), and three IKKε-induced genes (IFN-β, CCL3, and ISG15). The results show significant inhibition of expression of IKKε and of the RNA helicases RIG-I/MDA5/LGP2 in the HCV-infected patients, whereas expression of TBK1 and Cardif was not significantly altered. In conclusion, given the antiviral potential of IKKε and of the RNA helicases, these *in vivo* data strongly support an important role for these genes in the control of HCV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;44:1635-1647.)

Hepatitis C virus (HCV) infection leads to the development of chronic hepatitis in 60% to 90% of infected individuals, cirrhosis in 0.5% to 30% of cases, and hepatocellular carcinoma at a rate of 1% to 3% per year.[1] The current approved treatment for HCV infection is pegylated interferon alpha (IFN-α) in combination with ribavirin. This leads to clearance of the virus in 50% to 80% of cases, depending on the infecting HCV genotype. In particular, HCV of genotype 1 is the most resistant to IFN treatment.[2] HCV can interfere with the response of cells to IFN, through its viral proteins targeting either the IFN-activated JAK/STAT signaling pathway[3-5] or through other processes, leading to inhibition of action of the IFN-induced antiviral proteins.[6] In addition to this, HCV interferes with IFN induction, and more generally, with the induction of the innate immune response. The innate immune response is triggered in response to a variety of pathogens, such as bacteria and viruses, and is essential for a rapid limitation in the spread or action of these pathogens. IFN induction can take place after interaction of extracellular nucleic acids to members of the Toll-like receptor family[7-10] and after viral intrusion in the cytoplasm through the interaction of viral double-stranded RNAs (dsRNAs) with specific RNA helicases, such as RIG-I[11] or MDA-5.[12] For instance, the

Abbreviations: HCV, hepatitis C virus; IFN-α, interferon alpha; dsRNA, double-stranded RNA; NF-κB, nuclear factor-kappaB; RT-PCR, reverse transcription polymerase chain reaction; LPS, lipopolysaccharide.

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interaction of dsRNA with RIG-I provokes a change in its conformation that allows its N terminus CARD to trigger IFN induction[11,13,14] through association with the recently identified IPS-1/MAVS/VISA/Cardif protein.[15-18] The particularity of this latter adapter protein, referred to here as Cardif, is to localize to the mitochondrial membrane.[16] Cardif, in turn, recruits MAPKs, the IKK$\alpha\beta\gamma$ complex and the TBK1/IKKϵ kinases, thus provoking activation of nuclear factor kappaB (NF-κB) and IRF3[19,20] and, ultimately, IFN induction.[19,20]

The genome of the hepatitis C virus presents structured dsRNA regions, such as its 5′NTR and 3′NTR, which can trigger IFN-β induction soon after introduction in the cellular host.[21] However, HCV interferes with IFN induction through the action of its NS3/4A protease, which can cleave Cardif.[18,22,23] In addition, the NS3/4A protease can cleave the TRIF adapter, which links the IFN-inducing kinases to the dsRNA-activated TLR3,[24] thus emphasizing the importance of these two IFN signaling pathways and the necessity for the virus to inhibit them to favor its propagation.

The replication of HCV in the host may thus depend on a balance between its ability to abrogate both IFN induction and action and the ability of the cells to mount an efficient antiviral response. We have recently shown that the TBK1/IKKϵ kinases are not directly affected by the NS3/4A protease and that overexpression of IKKϵ in HCV replicon cells results in the inhibition of the HCV expression by 80%.[25]

In addition to phosphorylating IRF3 and inducing IFN, IKKϵ is also known to induce genes through the NF-κB and c/EBPδ transcription factors.[26-28] Therefore, to have exhaustive information on the involvement of IKKϵ in an antiviral action against HCV, we have analyzed its transcriptome profile after provoking its overexpression by transfection in cells expressing an HCV replicon. This revealed several genes involved in translational control, genes involved in apoptosis, and genes of the ISGylation pathway as well as genes from the chemokine family. Several of these genes are known to be induced directly through IRF3 and NF-κB, without the need for IFN induction, and, indeed, we could demonstrate that the antiviral response triggered by IKKϵ can develop in absence of IFN action. This shows that IKKϵ has the potential to provoke an immediate innate immune response against a viral intrusion. Furthermore, analysis of liver biopsies indicated a downregulation of the IKKϵ expression levels in the HCV-infected patients who were nonresponders to IFN treatment. Importantly, expression levels of RIG-I and MDA5, two RNA helicases responsible for IKKϵ activation, through Cardif, were found to be inhibited in all HCV-infected patients. This indicates that HCV may preferentially replicate and propagate from cells in which the IFN-inducing pathway is severely impaired.

Materials and Methods

Plasmids. The pcDNA3/AMP/zeo/flag (IKKϵwt) and pcDNA3/AMP/zeo/flag (IKKϵK38A) plasmids were described previously.[19,25] The pcDNA1/AMP and the pISRE-Luc were obtained from Invitrogen (Carlsbad, CA) and Stratagene (La Jolla, CA), respectively. The pRC/CMV flag STAT1α Y701F (Addgene plasmid 8702)[29] and pRC/CMV flag STAT1α (Addgene plasmid 8691)[30] were obtained from J Darnell through Addgene.

Antibodies. For neutralization of IFN-α binding, we used a purified preparation of anti-IFNAR1 monoclonal antibody 64G12 as described.[31,32] For the detection of total levels and phosphorylated levels of Stat1, we used polyclonal antibodies anti-Stat1, anti phosphoSTAT1 Ser727, and anti phosphoSTAT1 Tyr701 (Cell Signaling Technology, Danvers, MA). For the detection of flag STAT1α Y701F, flag STAT1α, and flag (IKKϵwt), we used anti-flag monoclonal antibody (anti-FLAG M2; Sigma, St. Louis, MO). For individual detection of IKKϵ, we used mouse anti-IKKϵ antibodies from BD Bio-Sciences (Franklin Lakes, NJ).[23] Protein loading control was performed with monoclonal anti-actin antibodies (Sigma).

Cell Culture. The Huh-7 and Huh-7 carrying the full-length HCV replicon 5.1 of genotype 1b were cultured as described.[25]

Transfection and Reporter Assay. Cells were transfected using lipofectamine 2000 (Invitrogen), and the reporter assays were performed as described.[25]

Real-Time RT-PCR Analysis. Total cellular RNA was extracted by acid-guanidinium thiocyanate-phenol chloroform using RNAble (Eurobio, Les Ulis, France) and according to the manufacturer's instructions. The sequence of the primers used for the reverse transcription polymerase chain reaction (RT-PCR) of GAPDH, IFN-β, IKKϵ, ISG15, ISG56, MDA-5, NOXA, and RIG-I were designed using the software LC Probe design (Roche, Basel, Switzerland) and choosing primers on each side of an intron (Supplementary Table 1; available at the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html). The sets of HCV primers[33] were designed as described.[33] (Supplementary Table 1). The following sets of primers were obtained commercially from the Qiagen (Valencia, CA) company (Human CCL3, gi: 48949814; Human CEBPδ, gi: 288772795; Human HECT E3, gi: 7705930; Human ISG 1-8U, gi: 11995467; Human

Table 1. Transcriptome Profile of the Top 50 Induced and the Total (24) Down-regulated Genes by IKKε in the Huh7 HCV Replicon Cells Using Affymetrix U133 2.0 Chips

AccNum	GeneName	Fold Change (log2)	Mean values IKKe	Mean values CONT
NM_002983	CCL3	4,09	9,46	5,37
BF514079	Kruppel-like factor 4 (gut)	3,48	8,88	5,39
NM_022873	ISG 6-16	3,31	9,67	6,36
NM_005101	ISG15	3,29	12,4	9,16
NM_022168	Mda-5	3,26	7,04	3,78
NM_001548	ISG56	3,25	9,94	6,69
BF338947	ISG 1-8 U	3,03	10,8	7,76
NM_006084	IRF9	2,98	10,1	7,1
U71088	MAPKK5	2,96	7,95	4,99
NM_016323	HECT E3 Ubiquitine ligase	2,92	8,29	5,38
NM_004585	RIG-I	2,81	8,05	5,24
M21121	RANTES	2,65	6,86	4,21
NM_002462	Mx1	2,64	8,56	5,92
NM_003733	2′–5′-oligoadenylate synthetase-like (OASL)	2,55	8,19	5,64
NM_002985	RANTES	2,54	7,97	5,43
BE888744	ISG54	2,48	8,32	5,84
NM_003641	ISG 9–27	2,44	8,27	5,84
NM_021127	NOXA	2,38	9,36	6,99
NM_002534	OAS	2,35	9,03	6,68
NM_016816	OAS 40/46kDa	2,32	8,26	5,95
AA749101	ISG 9–27	2,23	7,79	5,56
NM_001565	CXCL10	2,19	8,44	6,24
NM_006435	ISG 1–8 D	1,96	9,33	7,37
N47725	ISG58	1,95	6,83	4,89
AF063612	OASL	1,90	8,43	6,52
NM_006187	OAS 100kDa	1,90	8,57	6,67
AV755522	Unknown protein function	1,88	7,05	5,17
NM_021105	Phospholipid scramblase 1	1,82	7,9	6,08
NM_020995	Haptoglobin	1,78	9,97	8,18
AI825926	Phospholipid scramblase 1	1,77	9,83	8,06
NM_001549	RIG-G	1,77	7,87	6,1
NM_030641	Apolipoprotein L, 6	1,76	7,83	6,07
NM_021127	NOXA	1,63	8,18	6,55
NM_002155	Heat shock 70kDa protein 6 (HSP70B′)	1,58	8,2	6,62
NM_005143	Haptoglobin	1,52	10,3	8,75
NM_017414	Ubiquitin specific protease 18 (USP18)	1,50	8,46	6,96
NM_002201	ISG20	1,48	8,65	7,17
NM_006169	Nicotinamide N-methyltransferase	1,41	7,72	6,31
NM_001085	SERPIN 3A	1,39	9,94	8,56
NM_020119	Zinc finger CCCH type, antiviral 1	1,36	6,55	5,19
NM_001710	B-factor, properdin	1,33	8,84	7,51
NM_006018	G protein-coupled receptor 109B	1,31	8,17	6,85
AJ224869	chemokine (C-X-C motif) receptor 4	1,29	5,04	3,74
BC002704	STAT1	1,25	8,88	7,63
U88964	ISG20	1,24	8,61	7,37
M83667	CCAAT/enhancer binding protein (C/EBP), delta	1,22	10,8	9,55
AF002985	CXCL11	1,22	4,68	3,46
NM_002984	Chemokine (C-C motif) ligand 4	1,21	7,24	6,03
NM_007315	STAT1	1,20	11,1	9,85
NM_004223	Ubiquitin-conjugating enzyme E2L 6	1,18	11,1	9,95
NM_002952	Ribosomal protein S2	−0,03	14,2	14,2
NM_001402	Eukaryotic translation elongation factor 1 alpha 1	−0,06	14,1	14,1
AF070647	Actin related protein 2/3 complex, subunit 1A, 41kDa	−0,11	4,04	4,15
NM_006239	Protein phosphatase, EF hand calcium-binding domain	−0,15	3,17	3,32
AF077954	Protein inhibitor of activated STAT, 2	−0,19	4,67	4,86
NM_000216	Kallmann syndrome 1 sequence	−0,21	4,31	4,52
AL359602	Doublecortin and CaM kinase-like 2	−0,22	4,16	4,38
N30878	213780_at	−0,25	3,51	3,76
AL117520	216618_at	−0,25	3,7	3,95
NM_016300	220359_s_at	−0,28	3,58	3,86
NM_024697	Zinc finger protein 659	−0,29	3,56	3,85
AW974812	222325_at	−0,32	3,67	3,99
NM_024500	Sushi, von Willebrand factor type A	−0,33	3,49	3,83
NM_024087	Ankyrin repeat and SOCS box-containing 9	−0,35	6,76	7,11
D87077	KIAA0240	−0,37	5,97	6,33
AW968555	Transducin (beta)-like 1X-linked	−0,39	7,47	7,86
AI742626	HIV-1 Rev binding protein	−0,39	8,65	9,04
NM_003542	Histone 1, H4c	−0,39	12,3	12,7
NM_005325	Histone 1, H1a	−0,44	6,99	7,43
NM_002276	Keratin 19	−0,44	9,07	9,52
AA910371	Calreticulin	−0,54	10	10,6
BE857772	Ribosomal protein L37a	−0,55	8,62	9,18
X75296	HIR histone cell cycle regulation defective homolog A	−0,72	7,86	8,58
AK000847	Zinc finger protein 236	−0,92	8,38	9,29

NOTE. The accession number of the genes is given on the left, followed by the gene name. The numbers represent the fold change and the mean values (of triplicate samples) for IKKε-transfected and control HCV replicon cells. The full transcriptome profile (231 genes) is shown in Supplementary Material.

KLF4, gi: 31560548; Human RANTES, gi: 22538813; Human IFI-6-16, gi: 13259551). For each RT, the GAPDH antisense primer was included in each tube, for subsequent normalization. In the case of IKKε, IFN-β, and HCV, the RT was performed on 1 μg total RNA using each specific antisense primer and the rTth polymerase (Applied Biosystems, Foster City, CA) as described.[33] For the following genes: CCL3, CEBPδ, HECT E3, ISG15, ISG56, ISG 1-8U, IFI-6-16, KLF4, MDA-5, NOXA, RANTES, RIG-I, the RT was performed using the MuLV reverse transcriptase and oligo dT primers (Applied Biosystems). The cDNAs were then purified with the high pure PCR product purification kit (Roche Applied Science, Indianapolis, IN) in a final volume of 50 μL. Quantitative PCR was performed on a light cycler apparatus using SYBR Green, with 2 to 5 μL of the purified cDNA in a 10-μL reaction mixture containing 2 μL Light Cycler FastStart Plus DNA master SYBR Green Kit (Roche) and 0.5 μmol/L of each primer, as described.[33] Standard curves were established using 10-fold serial dilutions of: (1) plasmids containing the entire cDNA of the gene of interest (pcDNA3/AMP/zeo/flag (IKKεwt); (2) plasmids containing amplicons (GAPDH, IFN-β, ISG15, ISG56); (3) HCV synthetic plus strand RNAs; and (4) purified PCR products (CCL3, CEBPδ, HECT E3, ISG 1-8U, IFI-6-16, KLF4, MDA-5, NOXA, RANTES, RIG-I). The measured amounts of each mRNA were normalized to the amounts of GAPDH mRNA. The amounts of RNA were expressed as number of copies/μg total RNA.

Quantitative PCR of Liver Biopsies. Before RNA extraction, liver tissues were homogenized in 1 mL Ultraspec (Biotex, Houston, TX), and total RNA was obtained following the Ultraspec protocol. Two micrograms of the RNA preparations were submitted to DNAse (1 U/μL, Gibco-BRL, Grand Island, NY) in a total volume of 20 μL, for 15 minutes at room temperature, followed by inactivation at 65°C for 10 minutes in the presence of 2.5 μmol/L EDTA and 1 minute at 90°C. The reverse transcription was performed in 42 μL using 2.4 U Mu-MLV reverse transcriptase (Gibco-BRL) and 400 ng random primers (Roche) for 60 minutes at 37°C followed by 1 minute at 94°C. Quantitative PCR was performed as described above using 2 μL of the reverse transcription reaction and the primers for RIG-I, MDA5, LGP2, Cardif, TBK1, IKKε, CCL3, IFN-β, and ISG15 (Supplementary Table 1). For internal control genes, we used primers for three genes (TRIM44, HMBS, and BC002942) that have been determined to be the most stable and expressed at low levels in liver and are therefore the most representative genes to allow normalization of the samples[34] (Agnès Marchiato and Pascal Pineau, personal communication). For each liver biopsy, the arithmetic mean of the three Ct (Cycle treshhold) obtained for the internal controls was deduced from the Ct of the gene of interest to give a ΔCt value. These were then transformed to relative quantities using the formula $x^{\Delta Ct}$, where x represents the efficiency of PCR amplification in the Light Cycler apparatus (in our experiments, x = 1.9, representing an efficiency of 90%).

Immunoblot Analysis. Huh7 and Huh7 HCV replicon cells were seeded at 2×10^6 cells and at 2.2×10^6 cells/100-mm plates, respectively, and transfected after 24 hours using lipofectamine 2000 (Invitrogen). At 24 hours after transfection, the cells were scraped in their culture medium, pelleted by centrifugation, washed twice in phosphate-buffered saline, and the final pellet was either stored at −80°C before further processing or processed immediately. The cell pellets were resuspended in 400 μL lysis buffer (20 mmol/L HEPES,[35] 1% Triton X-100, 100 mmol/L NaCl, 1% aprotinin, 1 mmol/L PMSF) containing 1 mmol/L sodium orthovanadate (Na3VO4), 10 mmol/L β-glycerophosphate, and 50 mmol/L sodium fluoride (NaF) as phosphatase inhibitors as described in Tanabe et al.[36] After 20 minutes of incubation on ice, the cell extracts were centrifuged at 12,000*g* for 20 minutes at 4°C, transferred to other tubes, and stored at −80°C. The immunoblot analysis was performed as described.[25]

DNA Microarray Protocol. Total cellular RNAs were extracted by acid-guanidinium thiocyanate-phenol chloroform using RNAble (Eurobio) and further purified with an RNAeasy kit (Qiagen).The quality of RNAs was monitored on Agilent RNA Nano LabChips (Agilent Technologies, Santa Clara, CA). The reverse transcription (on 5 μg total RNA using OligodT primers), the *in vitro* transcription of the cDNA in presence of biotin, and the DNA fragmentation were performed according to Affymetrix standard protocols. Fragmented, biotin-labeled cRNA samples were hybridized on GeneChip® Human Genome U133A 2.0, containing probe sets representing 18,400 transcripts and variants including 14,500 well-characterized human genes, using Affymetrix standard protocols. For each sample, arrays were performed in triplicate, to minimize experimental variations. For each array, the cell intensity files (*.CEL) were generated with GeneChipOperating Software. Data analysis was performed using SPlus ArrayAnalyser software (Insightful). Data processing was done with the RMA method.[37,38] Statistical analysis to compare replicates arrays was done using the Local Pool error test.[39] The *P* values (the probability that the variability in a gene behavior observed between classes could occur by chance) were adjusted using Benjamini-Hochberg algorithm.[40] Upregulated genes

were distributed into clusters of cell functions using NetAffyx Gene Ontology Mining Tool software.

Results

Microarray Analysis of Genes Induced by IKKε in the Huh-7 Cells Expressing the HCV Replicon. Total RNAs were isolated 48 hours after transfection of Huh-7 cells expressing a full-length HCV replicon with either an IKKε expressing vector or an empty vector, used as control. HCV expression was inhibited in the IKKε-transfected cells as previously reported (not shown[25]). After purification, the mRNAs were reverse-transcribed using oligo-dT as primers and labeled with biotin after reamplification from the resulting cDNAs. They were then used as probes to hybridize 14,500 gene-expressing chips (Affymetrix; Affymetrix, Santa Clara, CA). This revealed a specific induction of 43 genes at or above twofold (base $\log_2$) and a limited number of 24 downregulated genes (Table 1) out of a list of 231 genes retrieved by the program (Supplementary Table 2). Specific induction was confirmed by qRT-PCR on 12 of the induced genes (CCL3, ISG15, MDA-5, KLF4, ISG6-16, ISG56, ISG 1-8U, HECT E3 Ubiquitin ligase, NOXA, RANTES, RIG-I, and C/EBPδ) (Fig. 1).

Ectopic Expression of IKKε in the HCV Expressing Cells Induces a Majority of Genes Linked to the IFN Antiviral Action. IKKε is functional in cells that have been subjected to lipopolysaccharide (LPS),[41] PMA,[42] dsRNA treatment, or viral infection[43] and is known to activate the IRF3, c/EBPδ, and NF-κB transcription factors.[19,20,26,44]

Accordingly, we observed that the IKKε transcriptome profile in the HCV replicon cells contains the genes MDA-5, ISG6-16, MX1, RIG-I, ISG9-27, ISG1-8D, ISG20, UbcH8, and the complement factor B, all genes that have been previously reported to be induced in response to a viral infection or dsRNA treatment. Similarly, it contains the genes IRF9, ISG58, PLSCR1, RIG-G, and STAT1, which are known to be induced by RA, LPS, or PMA (Table 2). More specifically, several genes of the IKKε transcriptome have been previously reported to be directly induced by IRF3. Those are: ISG15, ISG1-8, ISG56, ISG54, CXCL10, Viperin, NOXA, RANTES CXCL11, and USP18.[45-48] Induction of haptoglobin and antichymotrypsin ACT (SERPIN A3) by IKKε can be presumably attributed to c/EBPδ activation.[49,50]

Some of the genes of the IKKε transcriptome may be induced as secondary events through some transcription factors induced by IKKε. This might be the case for the KLF4 transcription factor, which is induced in the liver in response to IFNγ[51] and therefore could be induced here via STAT1, which is strongly induced by IKKε. Another example is that of the OAS genes.[52] Although those genes normally respond to the ISGF3 complex, resulting from the association of IRF9 with STAT1 and STAT2, here they may have been induced through IRF9 alone, because IRF9 overexpression has been previously noted to provoke the induction of several type I IFN-inducible genes.[53] The rest of the genes strongly induced by IKKε were previously associated with induction by STAT3 (MPK5, c/EBPδ) and by NF-κB, Sp1, HNF-1β, IRF1, or stress (Nicotinamide N-Methylase, ISG20, HSP70B, Vanin, MnSOD). With regards to NF-κB, IKKε was recently shown to phosphorylate p65 at two of its serine residues (468 and 536) and to control its nuclear import.[28]

In summary, the transcriptome profile of IKKε confirms the induction of already described genes and allows depiction of new IKKε-induced genes. In particular, these data point out that IRF3-induced genes and genes related to the antiviral action of IFN represent an important fraction of all the genes strongly induced by IKKε (Table 2).

The Protein Kinase IKKε Inhibits HCV Expression in the Absence of IFN Induction. IKKε can play an important role in IFN induction, via its ability to activate IRF3 and NF-κB. As many as 21 of the IKKε-induced genes in the HCV replicon cells are IFN-inducible genes (see Table 2); therefore IKKε may inhibit HCV expression through IFN induction and subsequent induction of a panel of genes with antiviral activity. Indeed, inhibition of HCV expression by IKKε was equivalent to that provoked by a concentration of IFN-α of between 1 and 10 U/mL (Fig. 2A). However, analysis of the microarray data indicates that several of the IKKε-induced genes, with known antiviral potential, may have been directly induced by IKKε, for instance, through IRF3 or NF-κB. To determine whether the antiviral effect of IKKε requires IFN induction, we then examined its effect on HCV expression in the presence of anti-IFNAR1 antibodies known to compete specifically for the binding of type I IFNs to the IFNAR chain of the IFN receptor.[31,32] Analysis of STAT1 phosphorylation in response to IFNα or IFNγ allowed to determine that the IFN receptor was functional in the HCV replicon cells as in the parental Huh-7 cells (Fig. 2B). The HCV replicon cells were then transfected with IKKε wt or the catalytically inactive mutant IKKε K38A and further incubated in presence of anti-IFNAR1 antibodies at a concentration sufficient to inhibit induction of an ISRE promoter-dependent luciferase reporter gene by 10 U/mL IFN-α (Supplementary Fig. 1). Both IKKε wt and IKKε K38A were expressed at similar levels, after transfection, as controlled by qRT-PCR (4-4.5 $\times$ 10^6 in the absence and 2 to 2.5 $\times$ 10^6 RNA

Table 2. List of Highly Induced Genes in the Huh7 Replicon Cells Upon IKKε Overexpression and Relation with Known Inducers

Locus link	Gene Name	Fold Change log2	Real change	Previously Described Inducers								Refs
				IRF3	c/EBP	NF-κB	Virus	dsRNA	Others	IFNαβ	IFNγ	
414062	CCL3	4.089	15.253		Yes	Yes					Yes	1.2
9636	ISG15	3.288	11.138	Yes			Yes	Yes	LPS	Yes		3
64135	MDA5	3.260	11.000							Yes		4
9314	KLF4	3.485	12.112								Yes	5
2537	ISG6-16	3.313	11.263				Yes	Yes	LPS	Yes	Yes	6
10410	ISG1-8	3.026	9.878	Yes			Yes	Yes		Yes		1.7
3434	ISG56	3.251	10.956	Yes			Yes		LPS	Yes		6
5607	MPK5	2.961	9.576						STAT3			8
10379	IRF9	2.982	9.675		Yes				RA	Yes		9,10,
51191	HERC5	2.916	9.365							Yes		11
5366	NOXA	2.375	6.967	Yes		Yes	Yes				Yes	1
4599	Mx1	2.635	8.094				Yes			Yes	Yes	12
6352	RANTES	2.645	8.139	Yes								13
5920	RIG-1	2.810	8.880					Yes	RA	Yes	Yes	14
8638	P59OASL	2.547	7.706							Yes		15
4938	OAS1	2.318	6.725							Yes		3
8519	ISG9-27	2.230	6.361				Yes	Yes		Yes		6
3433	ISG54	2.482	7.424	Yes								3
8519	ISG9-27	2.439	7.240					See above				6
4938	OAS1	2.350	6.863					See above				3
NM_002985	RANTES	2.541	7.681					See above				
3627	CXCL10	2.193	6.207	Yes			Yes					1
253635		1.881	4.975		Unknown					No protein function		
4940	OAS3	1.903	5.062							Yes		3
3240	Haptoglobin	1.523	3.669		ES (δ)							16
24138	ISG58	1.948	5.233						RA	Yes		17
5359	PLSCR1	1.823	4.757						PMA,RA	Yes		18
10581	ISG1-8D	1.961	5.284				Yes		LPS	NO	NO	6
7852	CXCR4	1.294	2.901									
5359	PLSCR1	1.769	4.556					See above				
80830	ApoL6	1.763	4.531									19
3240	Haptoglobin	1.523	3.669					See above				16
8638	P59OASL	1.905	5.066					See above				15
5366	NOXA	1.631	4.050					See above				1
1052	C/EBPδ	1.225	2.680						STAT3,Sp1			20
3437	RIG-G	1.766	4.542						PMA,RA	Yes		21
12	ACT	1.387	3.207						IL6,STAT3			22
6772	STAT1	1.200	2.601	NO					RA	Yes	Yes	23
3669	ISG20	1.479	3.518			Yes		Yes	IRF1	Yes	Yes	24
3310	HSP70B	1.584	3.882						Stress			
9246	UbcH8	1.184	2.551							Yes		25
6772	STAT1	1.247	2.751					See above				
6373	CXCL11	1.222	2.672	Yes		Yes						26
23586	RIG-I	1.102	2.301					See above				
8843	GpcR109B	1.313	2.964								Yes	27
4837	n.N.Methyl.	1.408	3.276						HNF-1 β			28
629	Compl.F.B	1.326	3.005					Yes				28
11274	USP18	1.503	3.599	Yes			Yes					30
55350	VANIN	1.103	2.305						Stress			31
55603	FAM46A	0.970	1.914					No information				32
91543	Viperin	1.127	2.378	Yes						Yes		33
6648	MNSOD	0.952	1.862			Yes			SP1			34

NOTE. Huh-7cells expressing a full length HCV replicon were plated in 60 mm at 7×10^5 cells/dish and transfected 24h after seeding with 3μg of an IKKε expressing vector or an empty vector, used as control. Each sample was performed in triplicate. At 48 hours after transfection, total RNAs were extracted with RNABle and processed for the micro-array analysis. This list shows 52 entries corresponding to genes which have been elevated around 2-fold or greater. Note that 9 genes are represented twice, which reduces the real number of highly induced genes to 4 3. The real change values are given next to those expressed in $\log_2$. The accession number of the genes is given on the left, followed by their usual name. Whenever possible, the genes were ascribed to their capacity to be induced by IRF3, c/EBP, NF-κB, three transcription factors known to be activated by IKKε, and more generally by virus, dsRNA or other stimuli of interest, using data retrieved from the literature. References are numbered on the right and given below:

(1)- C. P. Elco *et al, J Virol* **79** 3920 (2005); (2)-M. Grove, M. Plumb, *Mol Cell Biol* **13,** 5276 (1993); (3) T. Nakaya *et al., Biochem Biophys Res Comm* **283,** 1150 (2001); (4)- D. C. Kang *et al., Oncogene* **23,** 1789 (2004); (5)- Z. Y. Chen, *et al, Exp Cell Res* **281,** 19 (2002); (6)- P. M. Martensen, J. Justesen, *J Interferon Cytokine Res* **24,** 1 (2004); (7)- N. Grandvaux *et al., J Virol* **76,** 5532 (2002); (8) H. Song, *et al, Oncogene* **23,** 8301 (2004); (9)- X. Weihua, *et al, Biochim Biophys Acta,* **1492,** 163 (2000); (10)- S. Matikainen *et al., Cell Growth Differ* **8,** 687 (1997); (11)- A. Dastur, *et al, J Biol Chem* **281,** 4334 (2006); (12)- M. Aebi *et al., Mol Cell Biol* **9,** 5062 (1989); (13)- R. Lin, *et al, Mol Cell Biol* **19,** 959 (1999); (14)-T. Imaizumi *et al., Endothelium* **11,** 169 (2004); (15)- J. B. Andersen, *et al, Eur J Biochem* **271,** 628 (2004); (16)- T. Milosavljevic, *et al, Gen Physiol Biophys* **22,** 181 (2003); (17)- T. Niikura, *et al, Blood Cells Mol Dis* **23,** 337 (1997); (18)- K. W. Zhao *et al., Blood* **104,** 3731 (2004); (19)- Z. Liu, *et al, Mol Cancer Res* **3,** 21 (2005); (20)- G. S. Sivko, J. W. DeWille, *J Cell Biochem* **93,** 830 (2004); (21)-M. Yu *et al., Proc Natl Acad Sci U S A* **94,** 7406 (Jul 8, 1997); (22)- N. Kalsheker, *et al., Biochem Soc Trans* **30,** 93 (2002); (23)- L. H. Wong *et al., J Biol Chem* **277,** 19408 (2002); (24)- L. Espert *et al., Oncogene* **23,** 4636 (2004); (25)- C. Zhao, *et al, Proc Natl Acad Sci U S A* **102,** 10200 (2005); (26)- A. Marson, *et al., J Biol Chem* **279,** 28781 (2004); (27)- S. Tu naru *et al., Nat Med* **9,** 352 (2003); (28)- J. Xu, *et al, Mol Endocrinol* **19,** 527 (2005); (29)- J. Vandermeer, *et al, Arch Otolaryngol Head Neck Surg* **130,** 1374 (2004); (30)- O. Malakhova, *et al, J Biol Chem* **277,** 14703 (2002); (31)- T. K. Leung, *et al, Biochem J* **267,** 125 (1990); (32)- P. S. Lagali, *et al, Biochem Biophys Res Commun* **293,** 356 (2002); (33) K. J. Helbig, *et al, Hepatology* **42,** 702 (2005); (34)- D. K. St Clair, *et al, Methods Enzymol* **349,** 306 (2002).

copies in the presence of anti-receptor antibodies). In parallel, the Huh7 HCV Replicon cells were also incubated in presence of IFN, in the absence or presence of the anti-IFNAR antibodies. The results show that, although the antiviral action of IFN-α (90% inhibition of HCV expression) was significantly abrogated in the presence of anti-IFNAR1 (residual 27% of inhibition), the antiviral action of IKKε (86% in absence of antibodies) was only partially abrogated, with still 63% of inhibition of HCV expression (Fig. 2C).

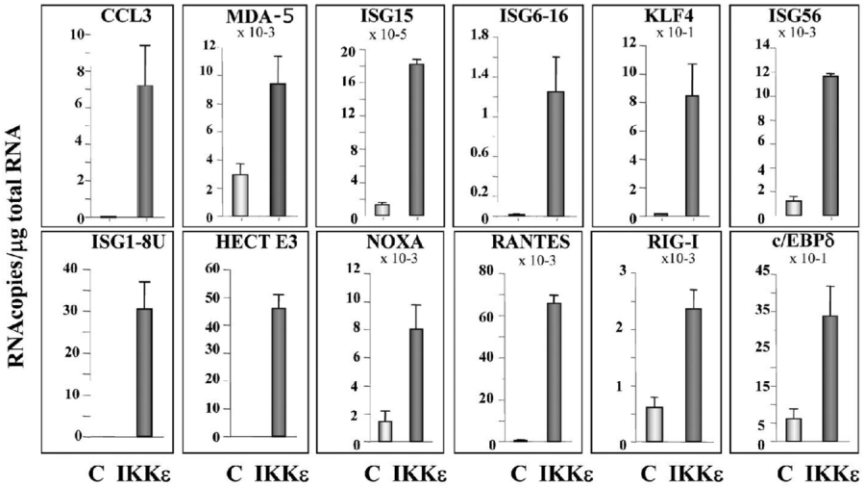

Fig. 1. Reverse transcription polymerase chain reaction (RT-PCR) validation of the micro-array analysis. The RNAs from two of the three samples used for the micro-array were reverse-transcribed using oligo-dT as primers. Individual assay by qPCR on 12 of the induced genes that were revealed by the micro-array analysis was performed as described in the Materials and Methods. The results are expressed in RNA copies/μg of total RNA.

As another readout to determine whether the IKKϵ-mediated ability to inhibit HCV expression requires IFN action, we have analyzed its effect in the presence of STAT1αY701F, a dominant negative form of STAT1α. Expression of this mutant prevents formation of any ISGF3 transcription factor complex (STAT1/STAT2/IRF9) that would occur on IFN activation of the JAK/STAT signaling pathway and induction of the IFN-stimulated genes.[54] Transfection of the Huh7 HCV Replicon cells with IKKϵwt again resulted in strong inhibition of HCV expression (81%), as shown in Fig. 2A,C. The HCV expression remained strongly inhibited when IKKϵwt was coexpressed with STAT1αY701F, with an increase in inhibition (88%). This further demonstrates that the antiviral activity of IKKϵ was independent of IFN production and action (Fig. 2D). In addition, we noticed that, although expression of the STAT1αY701F mutant alone had only a partial negative effect on HCV expression, similar to that of the IKKϵK38A mutant (35%), expression of a wild-type form of STAT1α proved to inhibit HCV expression to levels comparable to that of IKKϵ (Fig. 2D). This STAT1-mediated mechanism of HCV inhibition might be related to the ability of STAT1 to provoke apoptosis, for instance, through interaction with p53[55] (see Discussion). With regard to the IFN-independent antiviral activity of IKKϵ, overexpression of STAT1α (wt or Y701F) was reported to attenuate the IFN-α signaling, presumably by blocking IFN-α activated STAT2 translocation from IFNAR2 to IFNAR1.[56] Therefore, if the anti-HCV effect of IKKϵ had depended on IFN production, it would have been attenuated on STAT1α overexpression. The fact that IKKϵ still inhibits strongly HCV expression in presence of STAT1α confirms its independence, at least in part, from the necessity to produce IFN to generate an antiviral effect.

Therefore, these results show that the ability of IKKϵ to inhibit HCV can develop in the absence of IFN induction, probably through one or several of the genes identified by the microarray analysis.

The Expression of the Protein Kinase IKKϵ and of Some Other Components of the IFN Induction Pathway Are Downregulated in HCV-Infected Patients. We then analysed the *in vivo* expression levels of IKKϵ, to determine whether this protein kinase has the possibility to play an antiviral role in natural HCV infection. For this, we performed qRT-PCR on RNAs extracted from liver biopsies from nine HCV-infected patients (6 men, 3 women, aged 29-73 years) and eight HCV-infected patients who were nonresponders to IFN/ribavirin treatment (5 men, 3 women, aged 46-64 years) (Table 3). For controls, we used biopsies collected by laparectomy from tumor-free livers in 12 patients (8 men and 4 women aged 45-68 years); one patient had cholelithiasis, another hydatid cyst, and the remaining (10 patients) suffered from metastatic gastrointestinal cancer. To have a general picture of the expression of the IFN-inducing pathway, we also included in this study the analysis of the expression levels of the three RNA helicases: RIG-I, MDA5, and LGP2, the RIG-I/MDA5 adapter Cardif, TBK1, the second IRF3-phosphorylating kinase and three genes, downstream of the IFN-inducing pathway: IFN-β, CCL3, and ISG15.

We found that the relative expression levels of the components of the IFN-inducing pathway vary with the following order : Cardif $<$ RIG-I $<$ LGP2, TBK1 $<$ MDA5 $<$ IKKϵ, ISG15 $<$ IFN-β, CCL3, with Cardif and CCL3 having, respectively, the lowest and highest values (Fig. 3). Expression of CCL3 was increased in the HCV-infected patients, and this increase was statistically significant in the patient non-responders to IFN treat-

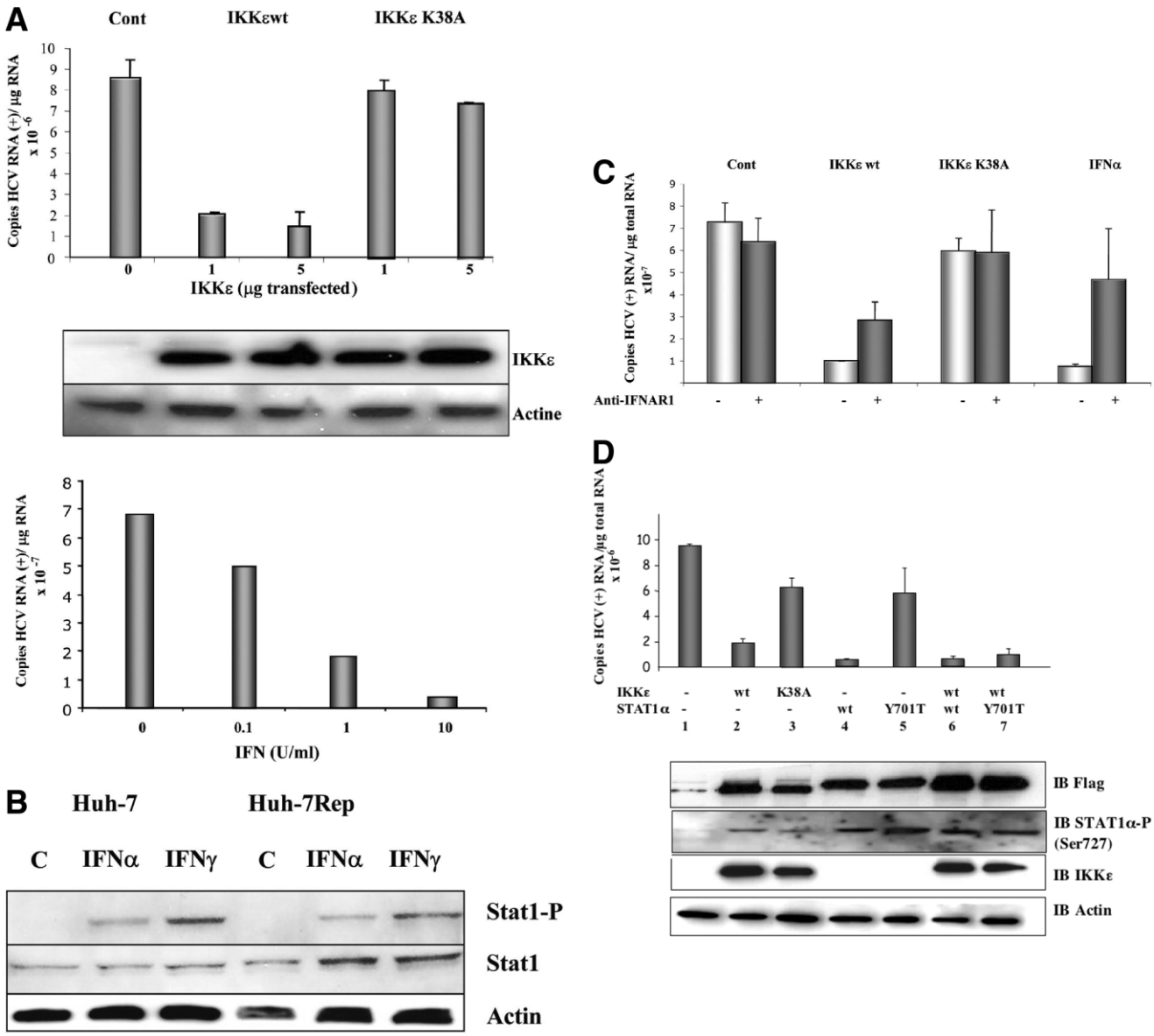

Fig. 2. IKKϵ inhibits hepatitis C virus (HCV) expression independently of IFN induction. (A) Huh-7 cells expressing a full-length HCV replicon were plated at 2×10^6 cells/100-mm dish and transfected after 24 hours with 5 μg pcDNA3/Amp/Zeo vector alone (0) or 1 and 5 μg of this vector expressing either flag-IKKϵ wt, or flag-IKKϵK38A (top). Three plates were prepared for each sample, of which two were used for the reverse transcription polymerase chain reaction (RT-PCR) analysis and one for the analysis of IKKϵ expression by immunoblot using anti-flag antibodies. In parallel, another set of these cells was treated with different concentrations of IFN-α (IFNαIFH, Le; Sigma) as indicated (bottom). RNA or protein extraction were performed at 48 hours after transfection or IFN treatment. In the case of RNA, the samples were processed for real-time RT-PCR analysis of HCV positive strand. The results are expressed in RNA copies/μg of total RNA. (B) Huh-7 cells and Huh-7 cells expressing a full-length HCV replicon were plated, respectively, at 2×10^6 cells and 2.2×10^6cells/100-mm dish. Twenty-four hours later, they were treated for 30 minutes with either 100 U/mL of IFN-α or 500 U/mL of IFNγ. Cells extracts were prepared and equivalent amounts of protein extracts (50μg) were used for immunoblot analysis using antibodies directed against full Stat1 or against a peptide containing the Stat1 phosphotyrosine 100. (C) Huh-7 cells expressing a full-length HCV replicon were plated at 7×10^5 cells/60-mm dish and transfected after 24 hours with 2 μg pcDNA3/Amp/Zeo alone (Cont) or 2μg either pcDNA3/Amp/Zeo (IKKϵwt) or pcDNA3/Amp/Zeo (IKKϵK38A). In parallel, a set of four dishes was also prepared for treatment with IFN-α. Three hours after transfection, 30 μg/μL of anti-IFNAR1 antibodies were added in the media of two dishes of each set. 10U/mL of IFNα was added in the appropriate dishes 45 minutes after addition of the anti-IFNAR1 antibodies. At 48 hours after transfection or IFN-α treatment, total RNAs were extracted with RNABle and the samples were processed for real-time RT-PCR analysis of HCV positive strand. The effect of IKKϵ or IFNα on HCV RNA expression was analysed in two independent experiments, and the results are expressed in RNA copies/μg of total RNA. (D) Huh-7 cells expressing a full-length HCV replicon were plated at 7×10^5 cells/60-mm dish (three dishes/sample) and transfected after 24 hours with 4 μg of pcDNA3/Amp/Zeo alone (lane 1), with 2 μg plasmids expressing IKKϵwt, IKKϵK38A, STAT1α, and STAT1α Y701F, respectively (lanes 2, 3, 4, and 5) or with 2 μg plasmid expressing IKKϵwt in presence of 2 μg of plasmid expressing either STAT1α wt (lane 6) or STAT1α Y701F (lane 7). At 48 hours after transfection, total RNAs were extracted with RNABle from two dishes of each sample and processed for real-time RT-PCR analysis of HCV positive strand. Two independent experiments were conducted (each performed in duplicate) and gave similar results. One of these two experiments is presented here,and the data are expressed in RNA copies/g total RNA. The third dish of each sample was processed for analysis of expression of IKKϵ and STAT1α by immunoblot using anti-flag antibodies. Because both IKKϵ and STAT1α constructs are tagged with flag, confirmation of the presence of STAT1 and IKKϵ in the doubly transfected cells was revealed with anti phosphoSTAT1 Ser727 and anti-IKKϵ antibodies.

Table 3. Clinical Data on Control and HCV-Infected Patients

Patient Number	ALT (IU/L)	AST (IU/L)	Viral Load (IU/mL)	HCV Genotype	Knodell Index	Treatment IFN/Ribavirin	Time After Treatment
1	78	29	5,00E + 06	1b	1 + 1 + 1 + 0	No	
2	31	18	3,45E + 05	1b	1 + 1 + 1 + 0	No	
3	75	47	2,36E + 05	4d	3 + 0 + 3 + 0	No	
4	93	54	2,02E + 05	1	1 + 3 + 4 + 0	No	
5	24	17	2,00E + 06	1b	0 + 1 + 1 + 0	No	
6	23	16	2,00E + 05	1a	NA	No	
7	70	34	1,00E + 06	4	1 + 1 + 1 + 0	No	
8	37	27	ND		1 + 1 + 3 + 1	No	
9	69	28	5,46E + 07	1a	1 + 1 + 1 + 0	No	
10	137	76	1,12E + 05	4	3 + 4 + 3 + 3	Yes	2 years
11	42	26	2,00E + 08	1b	NA	Yes	6 years
12	27	23	2,00E + 07	1b	1 + 1 + 3 + 1	Yes	9 years
13	47	32	1,20E + 07	1b	1 + 1 + 3 + 1	Yes	5 months
14	47	36	2,40E + 04	1b	1 + 1 + 3 + 0	Yes	4 years
15	70	41	5,91E + 05	1b	3 + 3 + 3 + 1	Yes	7 years
16	29	27	9,36E + 06	1a	1 + 1 + 3 + 0	Yes	5 years
17	36	25	4,00E + 05	1b	1 + 1 + 3 + 1	Yes	11 years

The ALT and AST normal values are <25 IU/mL and < 29 IU/mL respectively.

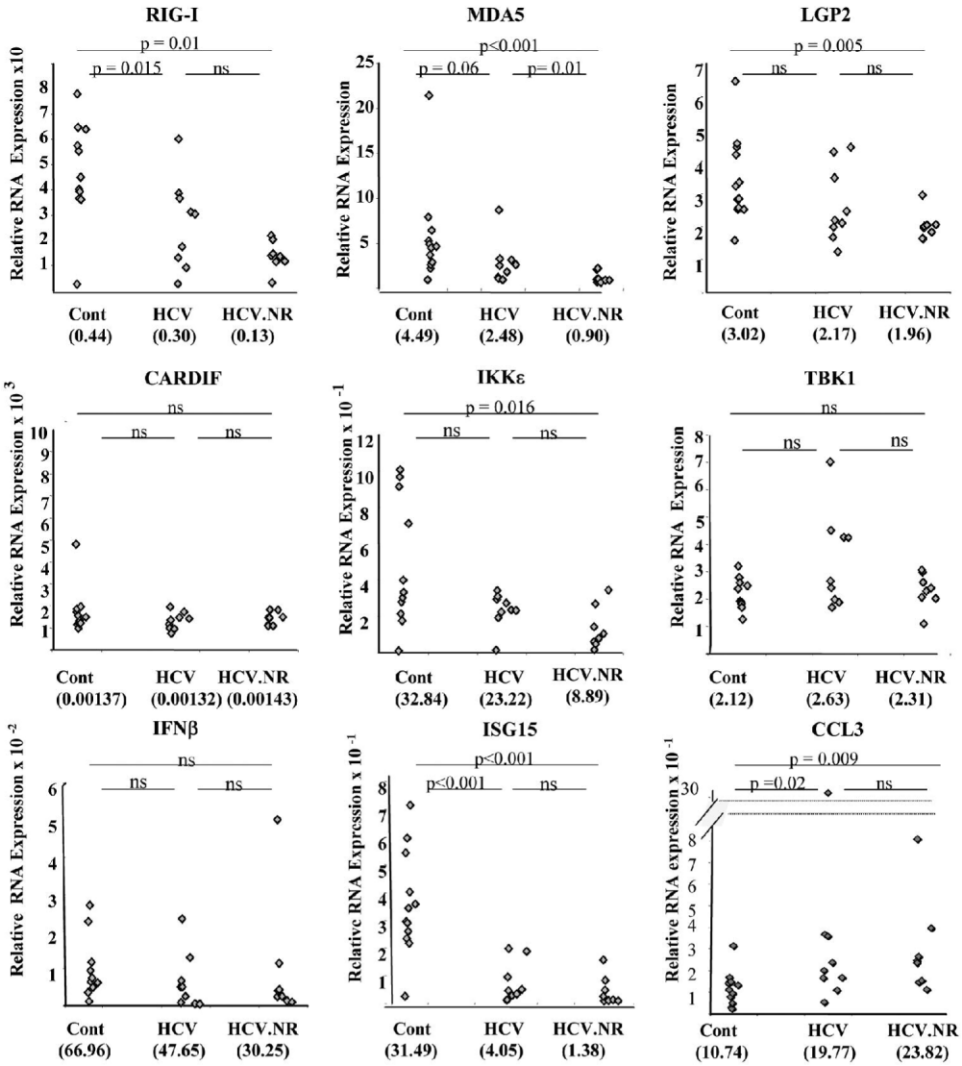

Fig. 3. Expression of IKKε and other genes of the IFN inducing pathway in liver biopsies from HCV-infected patients. Before RNA extraction, liver tissues were homogenized in 1 mL Ultraspec (Boitex, Houston, TX), and total RNA was obtained following the Ultraspec protocol. The RNA were then submitted to DNase treatment, and 2 μg RNA were reverse-transcripted with random primers. The samples were then processed for quantitative polymerase chain reaction (qPCR) analysis of the genes as indicated in the figure and as described in Patients and Methods. Statistical analyses were performed using non-parametric (Kruskal-Wallis and Mann-Whitney *U*) tests. All *P* values were two-tailed and considered significant if the associated value was less than .05 (NS = nonsignificant). SPSS 11.0 for Windows was used for the statistical analysis. Information on the uninfected patients (CONT), HCV-infected (HCV), and HCV-infected who have been nonresponders to IFN treatment (NR) is given under Table 3. For each gene, the median value of their relative expression level is given under each group.

ment as compared with the controls. CCL3 is a known marker of inflammation in hepatocytes,[57] and this increase therefore may reflect the inflammation state of the liver, associated with HCV infection. The IKKε protein kinase also can be induced in inflammatory states.[41,42] However, in contrast to the situation with CCL3, the IKKε expression levels did not increase in the HCV-infected patients and, in fact, even decreased with a significant decrease in the non responder patients as compared with the controls. This inhibition, thus, is indicative of some inhibitory effect on the IKKε expression attributable to the viral infection.

The expression levels of the three RNA helicases RIG-I, MDA5, and LGP2 were all downregulated in the nonresponder HCV-infected patients, and inhibition of RIG-I was already significant in the HCV-infected cohort. LGP2 is an RNA helicase related to RIG-I and MDA5, but devoid of CARD domain which has been reported to negatively control the activity of the two other RNA helicases through heterodimerization.[13] The expression levels of Cardif, TBK1, and IFN-β were not found to change significantly on HCV infection, whereas that of ISG15 was strongly inhibited. Altogether, the *in vivo* data collected from the analysis of the liver biopsies from HCV-infected patients show that the RNA helicases/IKKε pathway is particularly sensitive to the infection. A downregulation in the IKKε expression levels therefore may be detrimental for efficient induction of genes, such as ISG15. The decreased expression of IKKε, of the RNA helicases and of ISG15 cannot be attributed to HCV-associated cell death because all HCV patients presented moderate liver damage (Table 3). Coupled with the low expression levels of Cardif in the liver samples and the sensitivity of this protein to the HCV NS3/4A protease-mediated cleavage, these data confirm the importance of IKKε in the establishment of the antiviral state against HCV infection, because the inhibition of its expression by the infection can alter its efficacy to induce antiviral genes. Given the fact that IKKε was recently shown to strongly colocalize with Cardif,[23] these *in vivo* data confirm the *in vitro* observations that showed the importance of IKKε for the establishment of an ongoing antiviral state against HCV infection. The activity of this kinase in liver cells may be important to contribute to an equilibrium between the HCV infection and the protection of the cells and to limit the propagation of the virus.

Discussion

We previously showed that overexpression of the IKKε protein kinase in Huh7 cells expressing a full-length HCV replicon can inhibit the expression of this replicon. Here, we have established the transcriptome profile induced by IKKε in these HCV replicon cells. We showed that IKKε can induce expression of several genes that can play a role in antiviral action as well as in inflammatory responses and that its antiviral action still takes place in the presence of antibodies directed against IFNAR receptors, or in the presence of dominant negative STAT1α mutant. This indicates that an increase of IKKε expression levels may give the cells the possibility of resisting HCV infection.

The transcriptome profile induced by IKKε in the HCV replicon cells contains most of the genes involved in protein conjugation by the ubiquitin-like protein ISG15. In addition to ISG15, a known IRF3-induced gene,[58] the transcriptome contains the E2-(UbcH8) ligase and E3-CEB1/HERC5 ligase and the USP18 protease. Moreover, the IKKε transcriptome also contains the RIG-I, ISG56, ISG54, STAT1, and MxA genes, which can be ISG15 targets.[59] ISG15 forms covalent interaction with proteins but instead of targeting them for proteasomal degradation, like ubiquitin, it provokes posttranslational modifications that play a role on half-life, cellular localization, and protein activity. ISG15 conjugaison of proteins (or ISGylation) requires the activity of the E1-(Ube1L), E2-(UbcH8), and E3-CEB1/HERC5 conjugating enzymes,[59-61] whereas the deconjugation process is performed by the specialized ubiquitin protease USP18. The importance of USP18 in innate immunity to viral infection was demonstrated using USP18-deficient mice, which were resistant to lymphocytic choriomeningitis virus or vesicular stomatitis virus .[62] Although the exact antiviral activity of the ISG15 pathway remains to be determined, its induction by IKKε may play an important role in the antiviral action of IKKε against HCV.

The transcriptome induced by IKKε in the HCV replicon cells also revealed the expression of two apoptosis-mediating proteins of the BH3-only family: NOXA and the recently described apoL6.[63] They both belong to the pro-apoptotic group of the Bcl2 intracellular protein family,[64] and direct induction of NOXA by IRF3 has been recently reported.[48] Their induction may be of interest in view of the close association of IKKε with the mitochondria-associated Cardif[23] and a possible role for Cardif in the control of apoptosis.[16]

The protein kinase IKKε is strongly related to the other IRF3-phosphorylating kinase, TBK1. Studies with TBK1 and IKKε murine-deficient cells showed that TBK1 plays a major role in IFN-β induction in response to LPS, dsRNA (delivered intracytoplasmically), and virus infection. However, use of the IKKε/TBK1 doubly deficient cells revealed a complete abolition of IFN-β induction, and IKKε has been defined as an IRF3-phosphorylating kinase that could compensate, at least in part, for TBK1 deficiency in the $TBK1^{-/-}$ cells.[65] Both kinases can be

activated by Cardif; however, the mechanism of their activation is unclear, in particular their mode of interaction with Cardif. In one report, TBK1 was found to associate with Cardif and IKKε was not examined[17]; in another study, neither of these kinases was found to associate with Cardif but the data were presented only for TBK1.[15] In contrast, more recently, a strong association of Cardif with IKKε and no interaction with TBK1 was presented.[18] In accord with this latter result, recent confocal microscopy analysis demonstrated a tight colocalization for IKKε with the mitochondrial protein Cardif, whereas TBK1 was associated with other vesicles.[23] The Cardif/IKKε association could represent an important threat for HCV, which can be alleviated through the NS3/4A-mediated cleavage of Cardif.[18,22,23]

We showed that IKKε overexpression provoked inhibition of HCV expression. Based on these results, one might expect to find increased hepatic expression of IKKε in chronic hepatitis C as an adaptive response to halt HCV replication through the action of one or several of the antiviral molecules induced by IKKε. However, we found that the IKKε expression levels were downregulated in liver biopsies from HCV-infected patients, with an even more pronounced inhibition in the liver biopsies from patients who have been resistant to IFN treatment.

A decrease in the IKKε levels may create a situation favorable for HCV persistence. In addition to this, inhibition of the expression levels of the two RNA helicases RIG-I and MDA5 in the non responders HCV-infected patients, indicates that HCV infection can disrupt sufficiently the RNA helicase/IKKε chain to favor its propagation. RIG-I and MDA5 belong to the IFN-induced genes but even in case that induction of these genes might occur during IFN therapy, this is probably not sufficient to resist the infection because most HCV-infected patients are resistant to IFN/ribavirin treatment. A decrease in the IKKε levels may be a critical factor to explain HCV persistence and viral resistance to IFN because, as shown, IKKε mediates the induction of essential antiviral genes, including the two helicases, ISG15, enzymes, and targets of ISGylation, STAT1 among others.

The determination of the antiviral genes that are specifically induced by IKKε to provoke HCV inhibition obviously requires transcriptome profile analysis of IKKε-induced genes in HCV-infected TBK1$^{-/-}$ MEFs. This is at present not possible because the cellular models for HCV infection currently are restricted to human hepatoma–derived cell lines. However, we noticed that the following genes induced by IKKε in the HCV replicon cells: Mx1, OASL, apolipoprotein L, MDA5, ISG56, IP-10, Usp18, ISG15, CXCL11, CXCL-10, have been recently reported to be specifically induced by TBK1.[66] These genes therefore cannot be considered as specifically induced by IKKε, in normal conditions of activation of the innate immune response. Similarly, we found that the anti-HCV activity of IKKε persisted in presence of dominant negative STAT1 (Fig. 2D), which allowed ruling out of any STAT1-induced genes, such as KLF4, as candidate for a specific IKKε-induced antiviral response. However, STAT1 itself belongs to the IKKε gene profile in the HCV replicon cells and, interestingly, we observed that individual overexpression of STAT1wt resulted in a strong inhibition of HCV expression, similar to that provoked by IKKε (Fig. 2D). STAT1 has been reported to provoke apoptosis.[55] NOXA, an another important apoptosis-inducible gene,[67] was also highly induced by IKKε in the HCV replicon cells . Whether the antiviral activity of IKKε.against HCV is mediated through apoptosis remains to be determined.

Inhibition of IKKε expression in the HCV infected livers is intriguing because this gene can be induced through NF-κB, which is known to activate on HCV infection, for instance through the expression of the core and the NS5A proteins.[68] Moreover, IKKε can autoactivate and sustain its own induction, through C/EBPδ activation. One possibility is that IKKε activation may depend on its localization at the mitochondria in the vicinity of Cardif.[23] This information points to the importance of pursuing the generation of efficient and well-tolerated drugs that could abrogate the cleavage of Cardif during HCV infection.

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References

1. Zoulim F, Chevallier M, Maynard M, Trepo C. Clinical consequences of hepatitis C virus infection. Rev Med Virol 2003;13:57-68.
2. Hoofnagle JH. Course and outcome of hepatitis C. HEPATOLOGY 2002; 36:S21–29.
3. Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J Virol 1999;73:8469-8475.
4. Melen K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 2004;73:536-547.
5. Larrea E, Aldabe R, Molano E, Fernandez-Rodriguez CM, Ametzazurra A, Civeira MP, et al. Altered expression and activation of STATs (signal transduction and activator of transcription) in HCV infection: in vivo and in vitro studies. Gut 2006;55:1188-1196.
6. Pavio N, Lai MMC. The hepatitis C virus persistence: how to evade the immune system. J Biosci 2003;28:287-304.
7. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. 2000;408:740-745.
8. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-738.

9. Diebold S, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303:1529-1531.
10. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-1529.
11. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730-737.
12. Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, et al. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A 2004;101:17264-17269.
13. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, et al. Shared and unique functions of the DExD/H-Box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 2005;175: 2851-2858.
14. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19-28.
15. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 2005.
16. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF3. Cell 2005;122:1-14.
17. Xu LG, Wang Y-Y, Han K-J, Lii L-Y, Zhai Z, Shu H-B. VISA is an adapter protein required for virus-triggered IFN-b signaling. Mol Cell 2005;19:1-14.
18. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167-1172.
19. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300:1148-1151.
20. Fitzgerald KA, SM M, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nature Immunol 2003;4:491-496.
21. Sumpter R, Jr., Loo YM, Foy E, Li K, Yoneyama M, Fujita T, et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 2005;79:2689-2699.
22. Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A 2005;102: 17717-17722.
23. Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, et al. Dissociation of a MAVS/IPS/VISA/Cardif-IKke molecular complex from the mitochondrial outer membrane by hepatitis virus C NS3/4A proteolytic cleavage. J Virol 2006;80:6072-6083.
24. Li K, Chen Z, Kato N, Gale M, Jr., Lemon SM. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem 2005;280:16739-16747.
25. Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, et al. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. J Virol 2005;79:3969-3978.
26. Kravchenko VV, Mathison JC, Schwamborn K, Mercurio F, Ulevitch RJ. IKKi/IKKepsilon plays a key role in integrating signals induced by proinflammatory stimuli. J Biol Chem 2003;278:26612-26619.
27. Buss H, Dorrie A, Schmitz ML, Hoffmann E, Resch K, Kracht M. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. J Biol Chem 2004;279:55633-55643.
28. Mattioli I, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, et al. Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKKepsilon. J Biol Chem 2006;281:6175-6183.
29. Wen Z, Zhong Z, Darnell JE, Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241-250.
30. Horvath CM, Wen Z, Darnell JE, Jr. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. Genes Dev 1995;9:984-994.
31. Benoit P, Maguire D, Plavec I, Kocher H, Tovey M, Meyer F. A monoclonal antibody to recombinant human IFN-alpha receptor inhibits biologic activity of several species of human IFN-alpha, IFN-beta, and IFN-omega: detection of heterogeneity of the cellular type I IFN eceptor. J Immunol. 1993;150:707-716.
32. Cajean-Feroldi C, Nosal F, Nardeux PC, Gallet X, Guymarho J, Baychelier F, et al. Identification of residues of the IFNAR1 chain of the type I human interferon receptor critical for ligand binding and biological activity. Biochemistry 2004;43:12498-12512.
33. Castet V, Fournier C, Soulier A, Brillet R, Coste J, Larrey D, et al. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected in vitro. J Virol 2002;76:8189-8199.
34. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034.
35. Castelain S, Francois C, Bonte D, Baron A, Horle B, Morel V, et al. Epidemiological and quantitative study of GBV-C infection in french polytransfused children. J Med Virol 2004;73:596-600.
36. Tanabe Y, Nishibori T, Su L, Arduini RM, Baker DP, David M. Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. J Immunol 2005;174:609-613.
37. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249-264.
38. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-193.
39. Jain N, Thatte J, Braciale T, Ley K, O'Connell M, Lee JK. Local-pooled-error test for identifying differentially expressed genes with a small number of replicated microarrays. Bioinformatics 2003;19:1945-1951.
40. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001;125: 279-284.
41. Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Tatsumi Y, et al. IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IKappaB kinases. Int Immunol 1999;11:1357-1362.
42. Peters RT, Liao S-M, Maniatis T. IKKe is part of a novel PMA-inducible IkB kinase complex. Mol Cell 2000;5:513-522.
43. Hiscott J, Lin R, Nakhaei P, Paz S. MasterCARD: a priceless link to innate immunity. Trends Mol Med 2006;12:53-56.
44. Sankar S, Chan H, Romanow WJ, Li J, Bates RJ. IKK-i signals through IRF3 and NFkappaB to mediate the production of inflammatory cytokines. Cell Signal 2005;18:982-983.
45. Lin R, Heylbroeck C, Genin P, Pitha PM, Hiscott J. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. Mol Cell Biol 1999;19:959-966.
46. Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, et al. Gene induction pathways mediated by distinct IRFs during viral infection. Biochem Biophys Res Comm 2001;283:1150-1156.
47. Grandvaux N, Servant MJ, tenOever BR, Sen G, Balachandran S, Barber GN, et al. Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. J Virol 2002;76:5532-5539.

48. Elco CP, Guenther JM, Williams BRG, Sen GC. Analysis of genes induced by Sendai virus infection of mutant cell lines reveals essential roles of interferon regulatory factor 3, NF-kappaB, and interferon but not toll-like receptor 3. J Virol 2005;79:3920-3929.
49. Milosavljevic T, Lazic T, Uskokovic A, Petrovic M, Grigorov I. Expression of the rat liver haptoglobin gene is mediated by isoforms of C/EBPalpha, -beta and -delta proteins. Gen Physiol Biophys 2003;22:181-190.
50. Cardinaux JR, Allaman I, Magistretti PJ. Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. Glia 2000;29:91-97.
51. Chen ZY, Shie JL, Tseng CC. STAT1 is required for IFN-gamma-mediated gut-enriched Kruppel-like factor expression. Exp Cell Res 2002;281: 19-27.
52. Justesen J, Hartmann R, Kjeldgaard NO. Gene structure and function of the 2′-5′-oligoadenylate synthetase family. Cell Mol Life Sci 2000;57: 1593-1612.
53. Bluyssen HA, Muzaffar R, Vlieststra RJ, van der Made AC, Leung S, Stark GR, et al. Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. Proc Natl Acad Sci U S A 1995;92:5645-5649.
54. Improta T, Schindler C, Horvath CM, Kerr IM, Stark GR, Darnell JE, Jr. Transcription factor ISGF-3 formation requires phosphorylated Stat91 protein, but Stat113 protein is phosphorylated independently of Stat91 protein. Proc Natl Acad Sci U S A 1994;91:4776-4780.
55. Townsend PA, Scarabelli TM, Davidson SM, Knight RA, Latchman DS, Stephanou A. STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis. J Biol Chem 2004;279:5811-5820.
56. Radaeva S, Jaruga B, Kim WH, Heller T, Liang TJ, Gao B. Interferon-gamma inhibits interferon-alpha signalling in hepatic cells: evidence for the involvement of STAT1 induction and hyperexpression of STAT1 in chronic hepatitis C. Biochem J 2004;379:199-208.
57. Zhang T, Guo CJ, Li Y, Douglas SD, Qi XX, Song L, et al. Interleukin-1beta induces macrophage inflammatory protein-1beta expression in human hepatocytes. Cell Immunol 2003;226:45-53.
58. Au WC, Moore PA, Lowther W, Juang YT, Pitha PM. Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. Proc Natl Acad Sci U S A 1995;92:11657-11661.
59. Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. Proc Natl Acad Sci U S A 2005;102:10200-10205.
60. Yuan W, Krug RM. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. EMBO J 2001;20:362-371.
61. Dastur A, Beaudenon S, Kelley M, Krug RM, Huibregtse JM. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. J Biol Chem 2006;281:4334-4338.
62. Ritchie KJ, Hahn CS, Kim KI, Yan M, Rosario D, Li L, et al. Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. Nat Med 2004;10:1374-1378.
63. Liu Z, Lu H, Jiang Z, Pastuszyn A, Hu CA. Apolipoprotein l6, a novel proapoptotic Bcl-2 homology 3-only protein, induces mitochondria-mediated apoptosis in cancer cells. Mol Cancer Res 2005;3:21-31.
64. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 2005;17:617-625.
65. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, et al. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med 2004;199: 1641-1650.
66. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, et al. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol 2006;7:40-48.
67. Lallemand C, Blanchard B, Palmieri M, Lebon P, May E, Tovey MG. Single-stranded RNA viruses inactivate the transcriptional activity of p53 but induce NOXA-dependent apoptosis via post-translational modifications of IRF-1, IRF-3 and CREB. Oncogene 2006.
68. Liao QJ, Ye LB, Timani KA, She YL, Yang XJ, Ye L, et al. Hepatitis C virus non-structural 5A protein can enhance full-length core protein-induced nuclear factor-kappaB activation. World J Gastroenterol 2005;11:6433-6439.