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Frontline

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DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-β-inducing potential

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Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR) are members of the DEAD box helicases, and recognize viral RNA in the cytoplasm, leading to IFN-β induction through the adaptor IFN-β promoter stimulator-1 (IPS-1) (also known as Cardif, mitochondrial antiviral signaling protein or virus-induced signaling adaptor). Since uninfected cells usually harbor a trace of RIG-I, other RNA-binding proteins may participate in assembling viral RNA into the IPS-1 pathway during the initial response to infection. We searched for proteins coupling with human IPS-1 by yeast two-hybrid and identified another DEAD (Asp-Glu-Ala-Asp) box helicase, DDX3 (DEAD/H BOX 3). DDX3 can bind viral RNA to join it in the IPS-1 complex. Unlike RIG-I, DDX3 was constitutively expressed in cells, and some fraction of DDX3 is colocalized with IPS-1 around mitochondria. The 622-662 a.a DDX3 C-terminal region (DDX3-C) directly bound to the IPS-1 CARD-like domain, and the whole DDX3 protein also associated with RLR. By reporter assay, DDX3 helped IPS-1 up-regulate IFN-β promoter activation and knockdown of DDX3 by siRNA resulted in reduced IFN-β induction. This activity was conserved on the DDX3-C fragment. DDX3 only marginally enhanced IFN-β promoter activation induced by transfected TANK-binding kinase 1 (TBK1) or I-kappa-B kinase-ε (IKKε). Forced expression of DDX3 augmented virus-mediated IFN-β induction and host cell protection against virus infection. Hence, DDX3 is an antiviral IPS-1 enhancer.

Key words: DDX3 · IFN-β · IPS-1 · RIG-I-like receptors · Viral infection

See accompanying Commentary by Mulhern and Bowie

Introduction

Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are cytoplasmic RNA helicases [1–3], which signal the presence of viral RNA through the adaptor, IFN-β promoter stimulator-1 (IPS-1) (also known as mitochondrial antiviral signaling protein/caspase recruitment domain (CARD) adaptor inducing IFN-β (Cardif)/virus-induced signaling adaptor) to produce IFN-β [4–7]. IPS-1 localizes on the outer membrane of the mitochondria *via* its C-terminus [6]. Its N-terminus consists of a CARD domain, which interacts with the CARD domains of RIG-I and MDA5. Viral RNA resulting from penetration or replication are believed to assemble in the CARD-interacting helicase complex to activate the cytoplasmic IFN-inducing pathway. Although non-infected cells usually express minimal amounts of RIG-I/MDA5, the final output of type I IFN is efficiently induced at an early stage of infection to protect host cells from viral spreading.

Once IPS-1 is activated, the kinase complex consisting of TANK-homologous proteins and virus-activated kinases induce nuclear translocation of IFN regulatory factor-3 (IRF-3) to activate the IFN promoter [8]. NAK-associated protein 1, TANK-binding kinase 1 (TBK1) and I-kappa-B kinase-ε (IKKε) are components of the kinase complex that phosphorylates IRF-3 to induce type I IFN [9, 10]. RIG-I recognizes products of various RNA viruses, while MDA5 recognizes products of picornaviruses

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[1, 11]. RIG-I and MDA5 share the helicase domain, which is classified into the DEAD (Asp-Glu-Ala-Asp) box helicase family, and the domain can bind to various RNA structures. 5′-triphosphate RNA or short dsRNA is a ligand of RIG-I, whereas long dsRNA is a ligand of MDA5 [1, 12]. However, these RIG-I-like receptors (RLR) are usually up-regulated to a sufficient level secondary to IFN stimulation, suggesting that other molecular mechanisms are responsible for the initial sensing of viral RNA.

Here, we looked for molecules that bind IPS-1 by yeast two-hybrid, and found a DEAD box helicase, DDX3 (DEAD/H BOX 3), as a component of the complex of IPS-1. DDX3 facilitated IPS-1-mediated IFN-β induction to confer high antiviral potential on early infection phase of host cells. This is the first report showing that DDX3 is an IPS-1 complement factor for antiviral IFN-β induction in host infectious cells.

Results

Involvement of DDX3 in the IPS-1 complex

IPS-1 is constitutively present on the mitochondrial membrane and plays a central role in the cytoplasmic IFN-inducing pathway. We searched for proteins that bind IPS-1 in yeast. Using bait plasmids with the IPS-1 CARD region (aa 6–136), we screened a human lung cDNA library to isolate IPS-1 CARD-interacting proteins. We identified one clone, #62 that encodes the DDX3 C-terminal region (aa 276–662), which included partial DEAD box and helicase superfamily C-terminal regions (Fig. 1A). Their interaction was confirmed in HEK293FT cells by immunoprecipitation (IP), where DDX3 and IPS-1 were coupled (Fig. 1B). We confirmed that the C-terminal fragments of DDX3, at least 622-662 a.a, bound IPS-1 (data not shown). Taken together with the results of the yeast two-hybrid assay, the C-terminal portions of DDX3 directly bind the CARD-like region of IPS-1.

RIG-I and MDA5 helicases also bind the IPS-1 CARD domain [4]. In general, RNA helicases make a large molecular complex, and sometimes form homo- or hetero-oligomers. RIG-I binds to LGP2 helicase, and forms homo-oligomers during Sendai virus infection [11]. Hence, we examined whether DDX3 was associated with the RLR proteins by i.p. RIG-I and MDA5 co-precipitated with DDX3 (Fig. 2A), suggesting that DDX3 is involved in the complex of IPS-1 that interacts with RIG-I and/or MDA5. DDX3 bound the C-terminal helicase domain including the RD region of RIG-I (Fig. 2B). Thus, additional interaction may occur between DDX3 and RIG-I/MDA5. IPS-1 localizes to the membrane of mitochondria [6]. Three-color imaging analysis indicated that DDX3 in part co-localized to the IPS-1-mitochodria complex in non-stimulated resting HeLa cells, which express undetectable amounts of RLR (Fig. 2C and data not shown). These results together with accumulating evidence infer that non-infected cells harbor the complex of DDX3 and IPS-1 with minimal amounts of RIG-I/MDA5.

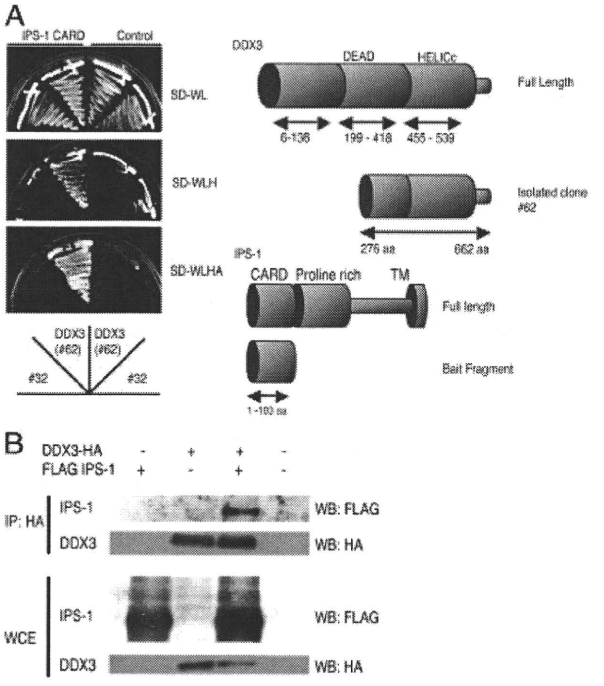

Figure 1. DDX3 binds IPS-1. (A) DDX3 partial cDNA fragment (aa 276–662) isolated by the yeast two-hybrid screening interacted with the IPS-1 CARD region (aa 1–103) in yeast. Tryptophan- and leucine-depleted synthetic dextrose medium plate (SD-WL) is non-selective, and tryptophan-, leucine- and histidine-depleted synthetic dextrose medium plate (SD-WLH) and tryptophan-, leucine-, histidine- and alanine-depleted synthetic dextrose medium (SD-WLHA) plates are selective plates. Empty bait plasmid (pGBKT7) was used for a negative control. (B) FLAG-tagged IPS-1 and HA-tagged DDX3 expression vectors were transiently transfected into HEK293FT cells by FuGeneHD reagent. 24 h after transfection, cell lysates were prepared, and IP was carried out using anti-HA Ab. The immunoprecipitates were analyzed by western blot using anti-HA or FLAG Ab. Data are representative of three independent experiments.

DDX3 promotes IPS-1-mediated IFN-β promoter activation

Forced expression of IPS-1 causes the activation of transcription from the IFN-β promoter. To ascertain the role of DDX3 in IFN-β production, we carried out reporter gene analysis to see the enhancing effect of DDX3 on IPS-1-mediated IFN-β promoter activation. Overexpression of DDX3 alone caused little activation of the promoter; however, the promoter activation was more augmented by minimal addition of DDX3 to IPS-1 than by overexpressed IPS-1 alone (Fig. 3A). This suggested that DDX3 enhanced IPS-1-mediated signaling despite the lack of RIG-I overexpression. To establish which region of DDX3 is important for IFN-β enhancer activity, partial DDX3 fragments were overexpressed with IPS-1, and IFN-β promoter activation was examined. The N-terminal region (aa 1–224, aa 224–487, aa 488–621) barely enhanced promoter activation (data not shown), but the C-terminal region (622–662) activated the promoter (Fig. 3B). These data indicated that the C-terminal region of DDX3 is important for the binding to IPS-1 and potentiation of the IPS-1 pathway.

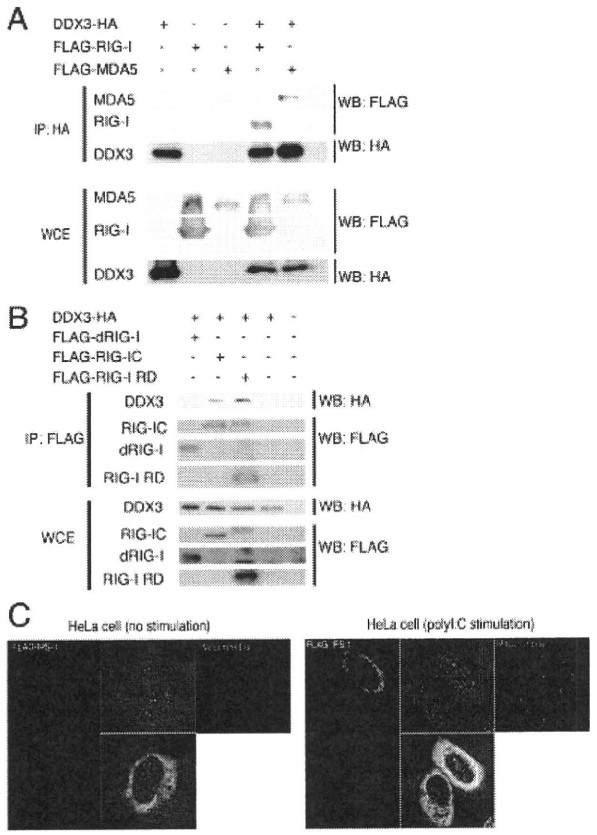

Figure 2. DDX3 joins the complex of RIG-I, MDA5 and IPS-1. (A) RIG-I and MDA5 co-precipitate with DDX3. HA-tagged DDX3 was expressed in HEK293FT cells, together with FLAG-tagged MDA5 or RIG-I, and 24 h after transfection, IP was performed using anti-HA Ab and analyzed by western blotting. (B) The C-terminal region of RIG-I participates in complex formation with DDX3. FLAG-tagged RIG-I fragments and HA-tagged DDX3 were expressed in HEK293 cells, and 24 h after transfection, IP was performed using anti-HA Ab and analyzed by western blotting. (C) DDX3 colocalizes with IPS-1. Flag-tagged IPS-1 and HA-tagged DDX3 were transfected into HeLa cells together with or without polyI:C. After 24 h, cells were fixed with formaldehyde and stained with anti-HA polyclonal and anti-FLAG monoclonal Ab. Alexa488 (DDX3-HA) or Alexa633 Ab was used for second Ab. Mitochondria was stained with Mitotracker Red. DDX-3 partially colocalized with IPS-1. Data are representative of three independent experiments.

DDX3 as a component of initial RNA sensor

RIG-I and MDA5 are IFN-inducible proteins, only traces of which exist in an early phase (<2 h) in the cytoplasm where viral RNA replicate. Previous reports showed that DDX3 binds RNA of poly rA or duplexed RNA [13, 14], and our protein analysis solidified this issue: DDX3 efficiently bound polyI:C and stem-loop RNA of viral origin in a solution (data not shown). DDX3 as well as IPS-1 were expressed even without any stimulation (Fig. 2C and 4A and B) and bound each other in the cytoplasm (Fig. 2C). Hence, DDX3 is a cytoplasmic molecule that can detect viral RNA produced in infected cells.

Knockdown studies suggested that polyI:C-mediated IFN promoter activation was abrogated in DDX3-deficient cells even in the presence of overexpressed RIG-I or MDA5 (Fig. 5). DDX3 silencing happened with two different siRNA. Thus, DDX3 may enable RIG-I and IPS-1 to confer activation of the cytoplasmic RNA-sensing pathway on virus-infected cells.

The IFN-β-inducing pathway involves IRF-3 kinases TBK1 and IKKε, which may be targets of DDX3 [15, 16]. By *in vitro* reporter analysis, increasing amounts of DDX3 barely affected IFN-β promoter activation by TBK1 and IKKε (Fig. 6A and B). Slight TBK1-enhancing activity could manage to be detected with DDX3 when decreasing amounts of TBK1 was used in the assay (Fig. 6C and D).

HeLa cells induced the mRNA of RIG-I and IFN-β in response to polyI:C stimulation within 1 h (Fig. 4A). More exactly, IFN-β induction was ~30 min faster than RIG-I induction in response to polyI:C. IFN-β mRNA induction was peaked around 3 h post stimulation, while RIG-I induction continued to increase >3 h (Fig. 4A). When HEK293 cells were infected with vesicular stomatitis virus (VSV) (a RIG-I-stimulating virus), the IFN-β mRNA was induced from 6 h, and by that time no RIG-I message was generated (Fig. 4B–D). The RIG-I message began to appear >8 h and was markedly increased (Fig. 4B and D). In either case, no up-regulation was observed with DDX3 but sufficiently present in the cytoplasm (Fig. 4C). Furthermore, overexpression of DDX3 in HeLa cells resulted in potential prevention of VSV propagation (Fig. 7). However, the distribution profiles of DDX3 and IPS-1 were barely altered in response to polyI:C stimulation (Fig. 2C). The results allow us to interpret that when viral RNA enter the cytoplasm of infected cells, the RNA first induce a small amount of IFN-β in conjunction with the complex containing trace RIG-I and then the induced IFN-β fosters intensive RIG-I/MDA5 induction. The complex is reconstituted together with upcoming RIG-I/MDA5 to amplify the cytoplasmic IFN-inducing pathway. Although the molecular reconstitution was not visible with overexpressed proteins by confocal analysis, DDX3 may act as an enhancing factor for initial RNA-sensing by the IPS-1 complex and conducts the rapid response to viral RNA to facilitate the IPS-1 signaling.

Discussion

We identified DDX3 as a protein that bound to the IPS-1 CARD region, duplexed RNA and RLR. Although the DDX3 helicase domain is a DEAD box type similar to those of RIG-I and MDA5, DDX3 does not have a signaling domain corresponding to the CARD domain. Therefore, DDX3 may not act as a signal sensor of RNA viruses, as RIG-I and MDA5 do. Considering the role of DDX3 in host RNA metabolism, it is more likely that DDX3 acts as a scaffold for RIG-I (even under the presence of low copy numbers of RIG-I) and intensifies IPS-1 signaling similar to LGP2 [11, 17]. RNA molecules usually form a complex with various

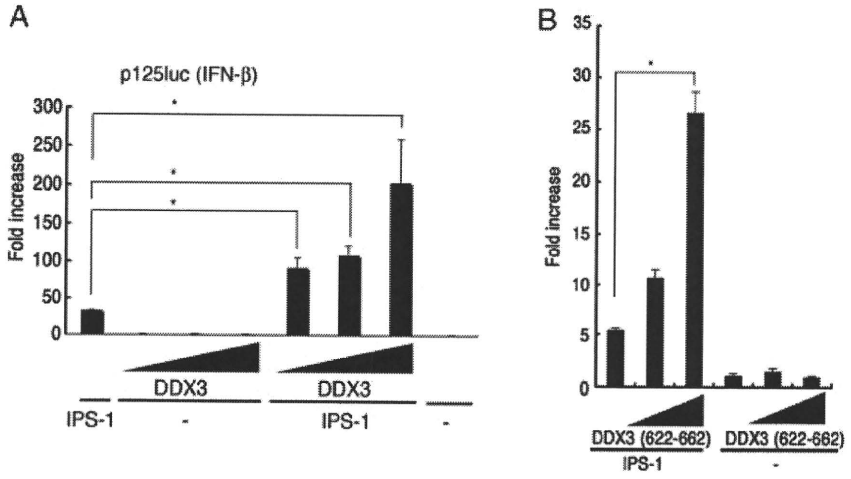

Figure 3. The C-terminal region of DDX3 participates in enhancing IPS-1-mediated IFN-β promoter activation. (A) Activation of IFN-β promoter was examined by reporter gene assay. HEK293 cells were transfected with DDX3- (100, 200 or 300 ng) and/or IPS-1 (100 ng)-encoding plasmids, together with reporter (p125luc) and control plasmids (Renilla luciferase) into 24-well plates. (B) The plasmids for expression of DDX3 (622-662 aa) and IPS-1 or the former only were transfected into HEK293 cells in 24-well plates together with p125luc reporter plasmid. After 24 h, the activation of reporter was measured. Data show mean fold induction+SD of three independent assays. $^*p<0.05$, Student's *t*-test.

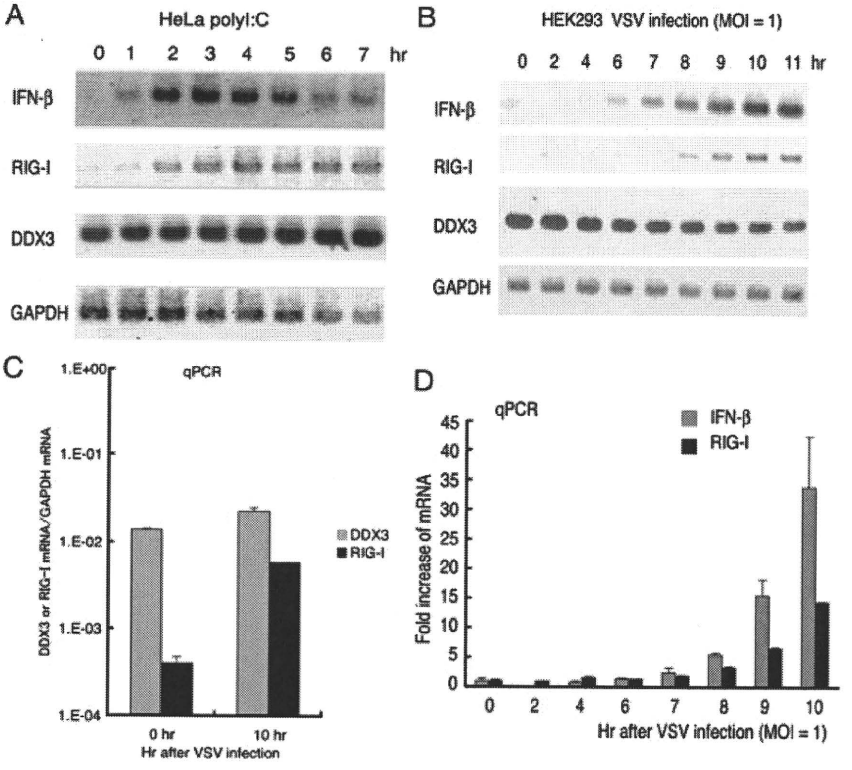

Figure 4. Earlier induction of IFN- than RIG-I in virus-infected cells. (A) Early induction of IFN-β in response to polyI:C. HeLa cells were stimulated with 50 μg/mL of polyI:C for indicated hours. Total RNA was extracted with TRIZOL and RT-PCR was carried out to examine the kinetics of expressions of DDX3, IFN-β, RIG-I and GAPDH (control). (B) IFN-β mRNA induction by VSV infection. HEK293 cell were infected with VSV at MOI = 1, and then total RNA was extracted with TRIZOL reagents at indicated times. The reverse transcription with random primers and PCR at 33 cycle were performed to detect RIG-I, DDX3 or IFN-β expression. Data are representative of three independent experiments. (C) Marked induction of RIG-I in VSV-infected cells. HEK293 cells were infected with VSV at MOI = 1, and then the total RNA was extracted with TRIZOL reagent at indicated times. The relative amounts of RIG-I or DDX3 mRNA were quantified by RT-qPCR, in which the mRNA of GAPDH was used for endogenous internal control. (D) Fold increase of IFN-β or RIG-I mRNA by VSV infection. The amount of IFN-β or RIG-I cDNA was determined by quantitative PCR. The fold increases were calculated by dividing the values of each time point by that of 0 h sample of IFN-β or RIG-I. Data show mean+SD pooled from three independent experiments.

proteins, such as 5′-end capping enzymes or translation initiation factors. Viral RNA also tends to couple with host proteins to replicate and translate RNA. DDX3 capturing RNA may function either in the molecular complex of RIG-I/MDA5/IPS-1 or in the complex of the translation machinery.

Recently, DDX3 was reported to up-regulate IFN-β induction by interacting with IKKε in the kinase complex [18]. IKKε is an NF-κB-inducible gene, whereas the DDX3-IPS-1 complex is constitutively present prior to infection. DDX3 may bind IKKε after IKKε is generated secondary to NF-κB activation [15]. Another report suggested that DDX3 interacts with TBK1 to synergistically stimulate the IFN-β promoter [16]. The report further suggested that DDX3 is recruited to the IFN promoter and acts like a transcription factor [16]. These reports also show that not C-terminal but N-terminal region of DDX3 is required for enhancing the IKKε- or TBK1-mediated IFN promoter activation. We showed that unlike these previous reports, the C-terminal region of DDX3 is important for the IPS-1 activation. These observations indicate that DDX3 is involved in RIG-I signaling at multiple steps. The involvement of DDX3 at several steps is not surprising, because DDX3 plays several roles in RNA metabolisms, such as RNA translocation or mRNA translation.

In cytoplasm, there are large amounts of DDX3 and only trace amounts of RIG-I in resting cells. Therefore, when the virus initially infects human cells, the viral RNA would encounter DDX3 before RIG-I capture the viral RNA. We demonstrated that the initial IPS-1 complex for RNA-sensing involves DDX3 in addition to trace RIG-I to cope with the early phase of infection. This IPS-1 complex activates downstream signal by involving a minute amount of viral RNA. What happens in actual viral infection is to first induce IFN-β and then RIG-I (Fig. 4B), suggesting that the initial IFN-β mRNA arises independent of the virus-induced RIG-I. Once IFN-β and RIG-I mRNA are up-regulated by viral RNA, the IPS-1 complex turns constitutionally different: the complex contains high amounts of RIG-I, which may directly capture viral RNA without DDX3. Our results indicate that the early IPS-1 complex formed in the early stages of virus-infected cells induce minute IFN-β with a mode different from the conventional IPS-1 pathway that RIG-I solely capture viral RNA and activates IPS-1. By retracting DDX3 from the complex by siRNA, only a minimal IFN-β response emerges merely with preexisting RIG-I and IPS-1, suggesting DDX3 to be a critical signal enhancer in the early IPS-1 complex. Development of a method to chase endogenous DDX3 will be required to test our interpretation.

The RIG-I generation occurring >8 h post RNA virus challenge makes the complex direct the conventional IFN-inducing pathway harboring sufficient RIG-I/MDA5. Previous reports [13, 14] and our RNA-binding analysis also speculated that one of the RNA-capture proteins is DDX3 since DDX3 tightly binds polyI:C and dsRNA in fluid phase. These RNA-capture proteins may have a role in the IPS-1-involving molecular platform in cells with early virus infection when only a trace RIG-I protein is expressed. This interpretation fits the result that DDX3 acts predominantly on an early phase of virus infection (Fig. 4B and 7).

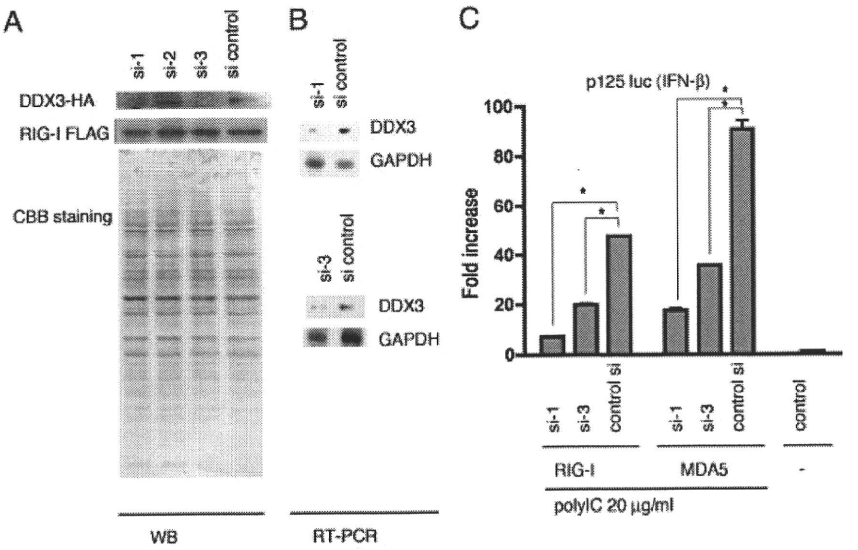

Figure 5. Knockdown of DDX3. (A) Negative control or DDX3 targeting siRNA (20 pmol), DDX3 si-1, -2 or -3, were transfected into HEK293 cells in 24-well plates, together with HA-tagged DDX3 or FLAG-tagged RIG-I expression plasmids, and after 48 h, cell lysates were prepared and analyzed by western blotting with anti-HA or anti-FLAG Ab, and the same membrane was stained with CBB. (B) DDX3 si-1, -3 or control siRNA was transfected into HEK293 cells, and after 48 h, expression of endogenous DDX3 mRNA was examined by RT-PCR. (C) DDX3 si-1, -3 or control siRNA was transfected into HEK293 cells with reporter plasmids and RIG-I- or MDA5 expression plasmid (100 ng). Forty-eight hours after transfection, cells were stimulated with polyI:C (20 μg/mL) with dextran for 4 h, and activation of the reporter was measured. siRNA for DDX3 reduced RIG-I- or MDA5-mediated p125luc activation. Data are representative of three independent experiments (A,B). Data show mean fold increase+SD pooled from three independent experiments (C). *$p<0.05$, Student's t-test.

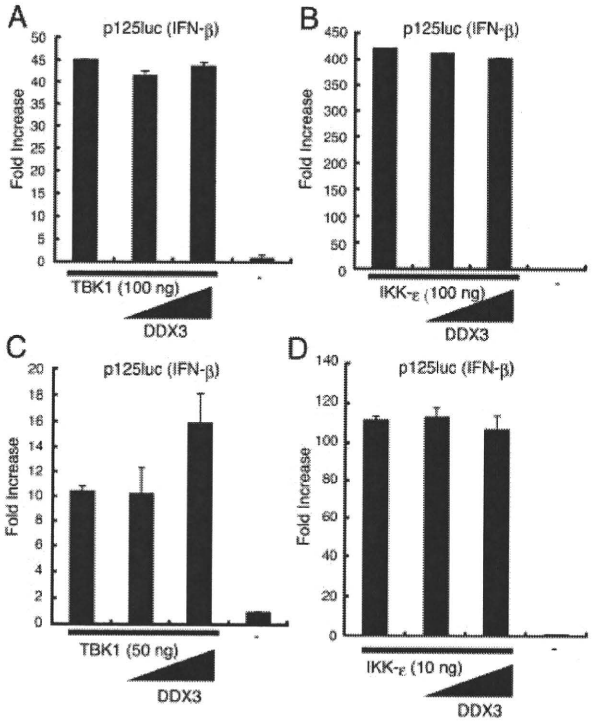

Figure 6. TBK1 and IKKε are not main targets for DDX3-mediated IFN-β up-regulation. (A–D) The activation of IFN-β promoter was examined by reporter gene assay. HEK293 cells were transfected in 24-well plates with DDX3 (0, 100 or 300 ng)-, TBK1 (0, 50 or 100 ng)- or IKKε (0, 10 or 100 ng)-encoding plasmid together with reporter (p125luc) and control plasmid. After 24 h, the cell lysate was prepared and the luciferase activities were measured. Data show mean+SD of three independent experiments.

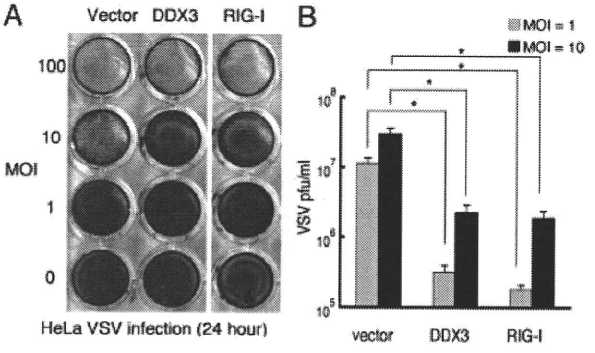

Figure 7. VSV infection is suppressed by overexpressed DDX3. (A) HeLa cells were transfected with DDX3, RIG-I or empty vector. After 24 h, the transfected cells were infected with VSV at indicated MOI. 24 h after VSV infection, the cells were fixed with formaldehyde and stained with crystal violet. (B) The VSV titers of culture supernatant of HeLa cells infected with VSV at MOI = 1 or 10 were measured by plaque assay. Data show mean+SD of three independent experiments. *$p<0.05$, Student's t-test.

Proteins involved in type I IFN induction are found ubiquitinated for their functional regulation. It has been reported that TRIM25 [19] and Riplet/RNF135 [20] act as ubiquitin ligases to activate RIG-I for IFN-β induction in their different sites of RIG-I ubiquitination. Another ubiquitin ligase RNF125 poly-ubiquitinates RIG-I through Lys48, leading to degradation of RIG-I [21]. The RIG-I level is highly susceptible to not only IFN but also ubiquitination in host cells. In addition, many viral factors may suppress the RIG-I function. It remains unknown what factor maintains a minimal level of RIG-I/MDA5 in resting cells. We favor the interpretation that DDX3 can be an alternative factor for compensating the low RLR contents in a certain infectious situation such that RIG-I is degraded or poorly up-regulated by other viral factors.

DDX3 is functionally complicated since its protective role against viruses may be modulated after the synthesis of viral proteins. DDX3 couples with the HCV core protein in HCV-infected cells and promotes viral replication [22]. This alternative function of DDX3 is accelerated by the HCV core protein, since the core protein withdraws DDX3 from the IFN-β-inducing facility, leading to suppression of IFN-β induction and positive regulation of HCV propagation in infected cells. DDX3 is also involved in HIV RNA translocation [14]. The DDX3 gene is conserved among eukaryotes, and Ded1 is a budding yeast homolog [23]. Ded1 helicase is essential for initiation of host mRNA translation, and human DDX3 can complement the lethality of Ded1-null yeast cells [24, 25]. Hence, another function of DDX3 is to bind viral RNA to modulate RNA replication and translocation. It is not surprising that DDX3 is implicated in various steps of RNA metabolism in cells with both host and viral RNA.

Materials and methods

Cell culture and reagents

HEK293 cells and HEK293FT cells were maintained in Dulbecco's Modified Eagle's low or high glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotics. HeLa cells were maintained in MEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS. Anti-FLAG M2 mAb, anti-HA polyclonal Ab, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor®-conjugated secondary Ab were from Invitrogen.

Plasmids

DDX3 cDNA encoding the entire ORF was cloned into pCR-blunt vector using primers, DDX3N F-Xh (CTC GAG CCA CCA TGA GTC ATG TGG CAG TGG AA) and DDX3C R-Ba (GGA TCC GTT ACC CCA CCA GTC AAC CCC) from human lung cDNA library. To make an expression plasmid, HA tag was fused at the C-terminal end of the full length DDX3 (pEF-BOS DDX3-HA). pEF-BOS DDX3 (1–224 aa) vector was made by using primers DDX3 N-F-Xh and DDX3D1 (GGA TCC GGC ACA AGC CAT CAA GTC TCT TTT C).

pEF-BOS DDX3-HA (225–662) was made by using primers DDX3D2-3 (CTC GAG CCA CCA TGC AAA CAG GGT CTG GAA AAA C) and DDX3C R-Ba. To make pEF-BOS DDX3-HA (225–484) and pEF-BOS DDX3-HA (485–663), the primers DDX3D2 R-Ba (GGA TCC AAG GGC CTC TTC TCT ATC CCT C) and DDX3D3 F-Xh (CTC GAG CCA CCA TGC ACC AGT TCC GCT CAG GAA AAA G) were used, respectively. Reporter and internal control plasmids for reporter gene assay are previously described [26].

RNAi

Knockdown of DDX3 was carried out using siRNA, DDX3 siRNA-1: 5′-GAU UCG UAG AAU AGU CGA ACA-3′, siRNA-2: 5′-GGA GUG AUU ACG AUG GCA UUG-3′, siRNA-3: 5′-GCC UCA GAU UCG UAG AAU AGU-3′ and control siRNA: 5′-GGG AAG AUC GGG UUA GAC UUC-3′. Twenty picomoles of each siRNA was transfected into HEK293 cells in 24-well plates with Lipofectamin 2000 according to manufacture's protocol. Knockdown of DDX3 was confirmed 48 h after siRNA transfection. Experiments were repeated twice for confirmation of the results.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as described previously [27]. The yeast AH109 strain (Clontech, Palo Alto, CA, USA) was transformed using bait (pGBKT7) and prey (pGADT7) plasmids. The transformants were streaked onto plates and incubated for 3–5 days. The IPS-1 CARD vector was constructed by inserting IPS-1 partial fragment encoding from 6 to 136 aa region into pGBKT7 multicloning site. Yeast two-hybrid screening was performed using human lung cDNA libraries. We obtained four independent clones, and one encoded DDX3 partial cDNA. SD-WLH is a yeast synthetic dextrose medium that lacks Trp, Leu and His aa. SD-WLHA lacks adenine in addition to Trp, Leu and His. SD-WL lacks Trp and Leu and thus non-selective plate.

Reporter assay

HEK293 cells (4×10^4 cells/well) cultured in 24-well plates were transfected with the expression vectors for IPS-1, DDX3 or empty vector together with the reporter plasmid (100 ng/well) and an internal control vector, phRL-TK (Promega) (2.5 ng/well) using FuGENE (Roche) as described previously [28]. The p-125 luc reporter containing the human IFN-β promoter region (−125 to +19) was provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). The total amount of DNA (500 ng/well) was kept constant by adding empty vector. After 24 h, cells were lysed in lysis buffer (Promega), and the *Firefly* and *Renella* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). The *Firefly* luciferase activity was normalized by *Renella* luciferase activity and is expressed as the fold stimulation relative to the activity in vector-transfected cells. Experiments were performed three times in duplicate (unless otherwise indicated in the legends).

PolyI:C stimulation

PolyI:C was purchased from GE Healthcare company, and solved in milliQ water. For polyI:C treatment, polyI:C (50 μg/mL) was mixed with DEAE-dextran (0.5 mg/mL) (Sigma) in the culture medium, and the cell culture supernatant was replaced with the medium containing polyI:C and DEAE-dextran. Using DEAE-dextran, polyI:C is incorporated into the cytoplasm to activate RIG-I/MDA5.

Virus preparation and infection

VSV Indiana strain or poliovirus type 1 Mahoney strain were used for virus assay. Vero derived cell (Vero-SLAM) was used for propagation and plaque assay for VSV indiana strain or poliovirus type 1 Mahoney strain. HEK293 cells were infected with viruses at MOI = 0.001 in a 24-well plate. The virus titers of culture media at indicated hours post infection in the figures were determined by plaque assay using Vero-SLAM cells. In some experiments that require rapid virus propagation, high MOI ($0.1 \sim 1$) was used for infection.

Immunoprecipitation

HEK293FT cells were transfected in a 6-well plate with plasmids encoding DDX3, IPS-1, RIG-I or MDA5 as indicated in the figures. Twenty-four hours after tranfection, the total cell lysate was prepared by lysis buffer (20 mM Tris-HCl (pH 7.5) containing 125 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 30 mM NaF, 5 mM Na3Vo4, 20 mM IAA and 2 mM PMSF), and the protein was immunoprecipitated with anti-HA polyclonal (Sigma) or anti-FLAG M2 mAb (Sigma). The precipitated samples were resolved on SDS-PAGE, blotted onto a nitrocellulose sheet and stained with anti-HA (HA1.1) monoclonal (Sigma), anti-HA polyclonal or anti-FLAG M2 mAb.

Confocal analysis

HeLa cells were plated onto cover glass in a 24-well plate. In the following day, cells were transfected with indicated plasmids using Fugene HD (Roch). The amount of DNA was kept constant by adding empty vector. After 24 h, cells were fixed with 3% of paraformaldehyde in PBS for 30 min, and then permeabilized with PBS containing 0.2% of Triton

X-100 for 15 min. For the polyI:C stimulation, 100 ng of polyI:C were transfected into HeLa cell in 24-well plates together with IPS-1 or DDX3 expressing vectors, and 24 h after the transfection, the cells were fixed and stained for confocal microscopic analysis. Permeabilized cells were blocked with PBS containing 1% BSA and were labeled with anti-Flag M2 mAb (Sigma), anti-HA polyclonal Ab (Sigma) or Mitotracker in 1% BSA/PBS for 1 h at room temperature. The cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with Alexa-conjugated Ab (Molecular Probes). Thereafter, micro-cover glass was mounted onto slide glass using PBS containing 2.3% DABCO and 50% of glycerol. The stained cells were visualized at × 60 magnification under a FLUOVIEW (Olympus, Tokyo, Japan).

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Abbreviations: **CARD:** caspase recruitment domain · **DEAD:** Asp-Glu-Ala-Asp · **DDX3:** DEAD/H BOX 3 · **IKKε:** I-kappa-B kinase ε · **IRF-3:** IFN regulatory factor-3 · **IP:** immunoprecipitation · **IPS-1:** IFN-β promoter stimulator-1 · **MDA5:** melanoma differentiation-associated gene 5 · **RIG-I:** retinoic acid inducible gene-I · **RLR:** RIG-I-like receptor · **TBK1:** TANK-binding kinase 1 · **VSV:** vesicular stomatitis virus

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医薬品が本業でないメーカーが独自技術を生かしワクチンの開発を進めている。東レはC型肝炎ワクチンを試作。森下仁丹は独自のカプセル技術で口から飲むタイプの腸チフスワクチンを開発する。新型インフルエンザの発生でワクチンに注目が集まるが、病気の流行状況などで需要が変動しやすい。参入組は安定需要が見込める分野に照準を定め、製薬会社と連携し実用化を目指す。

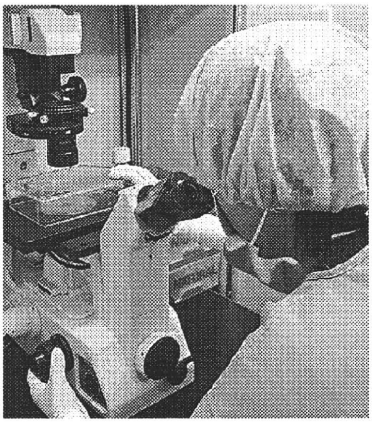
血液を介して感染し肝硬変になる恐れもあるC型肝炎。東レと国立感染症研究所などが試作したワクチンは、細胞を使う実験で日本人に多い「1b」型で66%、海外に多い「2a」型だと85%で感染を防げた。

C型肝炎ワクチンを作るには原因ウイルスを大量培養し濃縮・精製する必要があるが、有効な手法がなかった。感染研がウイルスを培養できる細胞を見つけたのを端緒に、東レがウイルスに目印をつけ効率よく濃縮する手法を開発。「ウイルスを1万倍に増やし、さらに1万倍に濃縮できる」（東レの中村紀子主任研究員）方法で実用化の展望が開けた格好だ。

同社にはC型肝炎治療用のインターフェロン製剤を超える可能性もある。量産するノウハウがあり、それをワクチン作りに活用する。患者は途上国を中心に毎年300万～400万人増え、世界で推定1億7千万人。市場規模はB型肝炎ワクチンの600億円を

技術ウオッチ

非医薬、ワクチンに触手



東レは独自技術を生かしC型肝炎ワクチンの実用化を目指す（国立感染症研究所）

▶ワクチン　人に備わる免疫機能を利用して感染症などを予防する。主に毒性を弱めたり、なくしたりした病原体をもとに作る。体の免疫系に病原体の特徴を伝え、病原体と闘う抗体を大量に作り出せるかが性能のポイント。注射タイプが多いが、途上国では投与しやすい経口型の潜在需要が大きい。

東レ、「肝炎用」開発にメド

日本では1980年代、集団予防接種時の事故で接種を控える人が増加。事業展望が読みづらくなり生産者もデンカ生研（東京・中央）など4社・団体にほぼ限られていた。だが海外製薬大手は予防医療の視点からワクチン事業の強化に動き、そこに独自技術を売り込む商機がある。

森下仁丹は神戸大学などと協力。「仁丹」で培ったカプセル技術を使い、飲む腸チフスワクチンの開発を進めている。

原因菌の遺伝子の一部をビフィズス菌に組み込み、カプセルに封入。胃酸に弱いビフィズス菌を腸まで運び、免疫系が抗体を作る。マウスの実験で治療に使える量の抗体ができることを確認。コレラや赤痢などへの応用も視野に入れる。

日油は感染研などとインフルエンザウイルスに効くワクチンを開発中だ。ウイルス自体に働く従来ワクチンと違い、感染した細胞を攻撃する。実現にはウイルスのたんぱく質を狙った場所に届ける技術が必要で、「優れた化学会社の協力が欠かせない」（感染研の内田哲也主任研究官）。

ただ承認を得るには大規模な臨床試験が欠かせず、日本ではワクチン全般で副作用を心配する人もいる。各社はまず海外の提携先を探す方針。東レの松田良夫主幹は「1～2年以内に相手を見つけ、臨床を見据えた動物実験に着手したい」と話す。

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