Rb/E2F1 Regulates the Innate Immune Receptor Toll-Like Receptor 3 in Epithelial Cells

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Tumor suppressor genes regulate the antiviral host defense through molecular mechanisms that are not yet well explored. Here, we show that the tumor suppressor retinoblastoma (Rb) protein positively regulates Toll-like receptor 3 (TLR3) expression, the sensing receptor for viral double-stranded RNA and poly(I-C). TLR3 expression was lower in Rb knockout (Rb^{-/-}) mouse embryonic fibroblasts (MEF) and in mammalian epithelial cells transfected with Rb small-interfering RNA (siRNA) than in control cells. Consequently, induction of cytokines interleukin-8 and beta interferon after poly(I-C) stimulation was impaired in Rb^{-/-} MEF and Rb siRNA-transfected cells compared to controls. TLR3 promoter analysis showed that Rb modulates the transcription factor E2F1, which directly binds to the proximal promoter of TLR3. Exogenous addition of E2F1 decreased TLR3 promoter activity, while Rb dose dependently curbed the effect of E2F1. Interestingly, poly(I-C) increased the Rb expression, and the poly(I-C)-induced TLR3 expression was impaired in Rb-depleted cells, suggesting the importance of Rb in TLR3 induction by poly(I-C). Together, these data indicated that E2F1 suppresses TLR3 transcription, but during immune stimulation, Rb is up-regulated to block the inhibitory effect of E2F1 on TLR3, highlighting a role of Rb-E2F1 axis in the innate immune response in epithelial cells.

Oncogenic virus infections, such as hepatitis C virus, human papillomavirus, Kaposi's sarcoma herpesvirus, and human T-cell leukemia virus 1 infections, are well-known causative factors of hepatocellular carcinoma, cervical carcinoma, lymphoma, and leukemia, respectively (30). These tumorigenic viruses attenuate tumor suppressor genes, activate proto-oncogenes, and consequently induce host cell abnormal growth. The tumor suppressor proteins p53 and Rb are frequently downregulated by oncogenic viral proteins during viral infection (11, 34, 51). Conversely, reactivation of p53 and Rb induces cell cycle arrest and apoptosis in virus-induced cancer (43, 61). Therefore, p53 and Rb are considered as critical proteins that prevent tumorigenesis caused by oncogenic viral infection (21, 30, 47). p53 activates interferon (IFN) signaling and induces apoptosis in infected cells by activating its target gene, the interferon regulatory factor 9 (IRF9) gene (36). p53 also increases viral sensing molecule Toll-like receptor 3 (TLR3) expression and function in human epithelial cells and mouse tissues (48), which correlates with the antiviral effect of p53 (35). Thus, p53 multiply regulates antiviral host defense, from sensing viral infection to signal transduction and viral removal by apoptosis, at the level of transcriptional regulation of p53 target genes (42).

The tumor suppressor Rb was identified as the protein responsible for the congenital tumor retinoblastoma, and this established the tumor suppressor paradigm for Rb in cancer research (8, 17, 37). Rb regulates cellular proliferation by directly binding to E2F transcription factors (9, 16, 45), a family of transcription factors that play a pivotal role in the regulation of cellular proliferation, growth, and differentiation (39, 58). The Rb-E2F binding negatively affects E2F-dependent transcription by at least three independent mechanisms: (i) masking the E2F's transactivation do-

main, (ii) sequestering the E2F from target promoters, and (iii) recruiting chromatin-modifying repressive complexes (18, 19, 23). The functional loss of Rb promotes the deregulated E2F activity that is observed in vast majority of human tumors (22, 25). Although the Rb-E2F pathway is well known to have a generally crucial role in oncogenesis, its functions during viral infection and antiviral host defense are not well studied. The founding member of the E2F family, E2F1, is mostly known for its transcriptional activating functions but, intriguingly, recent studies have revealed that E2F1 may act as a repressor as well. E2F1 was found to suppress, directly and independently of Rb, the promoters of vascular endothelial factor A (31), human telomerase reverse transcriptase (12), the antiapoptotic protein Mcl-1 (13), endoplasmic reticulum chaperone GRP78/Bip (41), and IRF3 (55). These surprisingly diverse gene targets of E2F1 revealed a wide influence of E2F1 on cellular events not only by activating but also by suppressing gene expression.

Double-stranded RNA (dsRNA) is an intermediate component during viral replication in host cells that is recognized by host innate immune molecules, including TLR3, protein kinase recep-

Received 19 October 2011 Returned for modification 22 November 2011

Accepted 25 January 2012

Published ahead of print 6 February 2012

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doi:10.1128/MCB.06454-11

TABLE 1 Primers used for real-time quantitative RT-PCR

Protein	Primer sequence (5'–3')	
	Sense	Antisense
Human		
E2F1	GCCACTGACTCTGCCACCATAG	CTGCCCATCCGGGACAAC
Rb	AGGATCAGATGAAGCAGATGG	TGCATTGCTGTTTCGAGTAGAAG
MDA5	TCCAACCTGCTGAACCTCCT	TGCCCATGTTGCTGTTATGT
RIG-I	CAGTATATTCAGGCTGAG	GGCCAGTTTTCTTGTGTC
PKR	ACTTTTCTCTGGCTCATCTC	ACATGCCTGTAATCCAGCTA
Mouse		
KC	TGTCAGTGCCTGCAGACCAT	CCTCGCGACCATTCTTGAGT
IFN- β	CACCTGAAGAGCTATTACTGG AGGG	CTCGCACCACCATCCAGG

tor (PKR), RIG-I, and MDA5 (32). These sensing molecules induce immune responses such as cytokine and chemokine production, which is an integral part in the initial action of antiviral host defense in both innate and adaptive immunity (1, 5). TLR3 and PKR transcription is induced by p53, which initiates the signaling cascade of antiviral host response (48, 59). Adversely, viral dsRNA suppresses p53 expression (29). These reports highlighted the correlation between tumor suppressor genes and dsRNA sensing. In the present study, we found that Rb, via E2F1, also critically impacts on TLR3 expression and signaling in epithelial cells. Rb knockout mouse embryonic fibroblasts (MEF), as well as cells transfected with Rb small interfering RNA (siRNA), showed decreased TLR3 expression. On the other hand, E2F1 negatively regulated TLR3 expression in epithelial cells. TLR3 ligand poly(I:C)-induced cytokine response was attenuated in the absence of Rb. Moreover, we found that poly(I:C) stimulation increased Rb, but not p53 expression, and inhibited E2F1 recruitment to TLR3 proximal promoter region, resulting in increased TLR3 transcription. Based on our findings, we identified Rb as a novel TLR3 regulator that affects viral host defense.

MATERIALS AND METHODS

Reagents and antibodies. Poly(I:C) was purchased from InvivoGen (San Diego, CA). Antibodies for Rb (sc-102), E2F1 (sc-251, sc-193), IRF-3 (sc-9082), normal mouse IgG (sc-2025), and γ -tubulin (sc-7396) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated Rb (Ser780; catalog no. 9307), I κ B- α (catalog no. 9242), phosphorylated I κ B- α (14D4), and phosphorylated IRF-3 (4D4G) were purchased from Cell Signaling Technology (Danvers, MA). Anti-TLR3 antibody (MAB1487) was purchased from R&D Systems (Minneapolis, MN). Anti-Hsc-70 antibody (SPA-815) was obtained from Stressgen Bioreagents (Canada). The horseradish peroxidase-conjugated secondary antibodies used in the present study were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell culture, treatment, and transfection. Human colorectal cancer cell line HCT116 (p53^{+/+} or wild-type) and HCT116 p53^{-/-} cells (7)

were kindly provided by B. Vogelstein from Johns Hopkins University. Lung adenocarcinoma cells (A549), cervical carcinoma cells (HeLa), and human hepatoma cells (HepG2) were obtained from the American Type Culture Collection. These cells were cultured and maintained as described previously (50). MEF from WT and Rb^{-/-} mice were prepared from E12.5 embryos as reported previously (46). Poly(I:C) treatment was carried out at the indicated time and concentration. At the time of treatment, the culture medium was replaced with fresh, serum-free medium containing poly(I:C) as described previously (49). Transient transfections of plasmids were performed using Trans-IT LT-1 (Mirus, Madison, WI) according to the manufacturer's recommendation. siRNAs for Rb and E2F1 were transfected into HCT116 cells using Trans-IT TKO (Mirus) according to the manufacturer's instructions. Concentrations of 50 or 100 nM Rb siRNA or E2F1 siRNA duplex were transfected into 70% confluent cells to knock down Rb and E2F1, respectively. GL2-luc siRNA duplex was used as a control. The cells were harvested 48 h after transfection. The oligonucleotide sequences for E2F1 siRNA and GL2 siRNA were shown previously (50). The sequences for the Rb siRNAs were 5'-GAUACCAGAUC AUGUCAGATT-3' (sense) and 5'-UCUGACAUGAUCUGGUAUCTT-3' (antisense).

Real-time RT-PCR analysis. Total RNA was isolated from cells using RNeasy Plus (Takara, Japan) according to the manufacturer's instructions. Real-time quantitative reverse transcriptase PCR (RT-PCR) analyses were carried out with SYBR green Master Mix (Applied Biosystems, California). PCR amplifications were performed as described previously (48). The threshold cycle (C_T) values for each gene amplification were normalized by subtracting the C_T value calculated for 18S rRNA (internal control). The normalized gene expression values were expressed as the relative quantity of gene-specific mRNA. The oligonucleotide primers used in the real-time quantitative PCR amplifications for human and mouse TLR3, human and mouse interleukin-8 (IL-8), and human IFN- β (48) and the primers for human and mouse 18S rRNA (50) were as described previously. The quantitative RT-PCR primers for human Rb, E2F1, MDA5, RIG-I, and PKR and mouse IL-8/KC and IFN- β are listed in Table 1.

Plasmids and luciferase assay. The cloning of TLR3 promoter constructs (bp -2006/+48, -987/+48, and -504/+48) in the luciferase reporter vector, pGL3-Basic vector (pGL3b; Promega, Madison, WI), was described previously (48). TLR3 promoter constructs containing mutation(s) in E2F binding sites were prepared by using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the recommended protocol. The primers used for generating TLR3 mutant promoters are listed in Table 2. The pSG5-Rb expression plasmid was kindly provided by Mark E. Ewen of Tufts University. The E2F1 expression plasmid was from W. Sellers (Addgene, plasmid 10736).

For luciferase assays, HCT116 cells seeded onto 12-well plates were transfected with 0.2 μ g of TLR3-luc promoter construct, together with the *Renilla* luciferase plasmid (pHRG-TK; Promega Corp. Madison, WI), Rb and the E2F1 expression plasmid. pSG5 and pcDNA3.1 empty vector were used as controls. The luciferase activity was determined by using a dual-luciferase reporter assay system (Promega) according to the manufacturer's recommended protocol.

ChIP assay. To examine the binding of E2F1 to TLR3 promoter, the nuclear extracts of HCT116 cells untransfected (for endogenous binding) or overexpressed with E2F1 were used for chromatin immunoprecipitation.

TABLE 2 Primers used for the generation of E2F-mutated 0.5-kb TLR3 promoter

Primer	Sequence (5'–3')
TLR3 prom MT1_sense	CTTTGCCCTTCTTGAATTACCAGACATAAAAGCAT
TLR3 prom MT1_antisense	ATGCTTTTATGTCTGGTGAATTCGAAGAAGGGCAAAG
TLR3 prom MT2_sense	CCTCTCAGCTCTGCCATGTCTGGCTCTCTCTGCTGTG
TLR3 prom MT2_antisense	CACAGCAGAGAGAGAGCCAGACATGGCAGAGCTGAGAGG
TLR3 prom MT3_sense	ACTCAATTCTGACAGCTACACATGAGTCTAGCAGAAAA
TLR3 prom MT3_antisense	TTTCTGCTAGACTCATGTGTAGCTGTGAGAATTGAGT

FIG 1 Rb increases TLR3 expression in epithelial cells. (A) TLR3 mRNA was examined in WT MEF and Rb^{-/-} MEF by quantitative PCR. (B and C) HCT116 cells were transfected with si-Rb or si-GL2 (control) (B) or with 0.5, 1.0, or 2.0 μg of Rb expression plasmid or control vector (C). Total RNA from these cells was isolated for analysis of TLR3 mRNA by quantitative PCR. (D and E) TLR3 protein was examined in HCT116 cells transfected with Rb expression plasmid (D) or with si-Rb or si-GL2 (E). (F to L) TLR3 (F to H) and Rb (I to L) mRNA levels were assessed in the indicated epithelial cell lines transfected with si-Rb or si-GL2. For quantitative PCR analyses, mRNA expression was normalized to mouse or human 18S rRNA. Values are means ± the SD ($n = 3$). For panels A, B, and F to L, the P values were assessed by unpaired t test. For panel C, the P values were assessed by ANOVA with Dunnett's test. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively, versus the control. n.s., not significant.

tion (ChIP) assay according to the protocol described previously (48). First, 2 μg of anti-E2F1 antibody or normal rabbit IgG was incubated with precleared chromatin. Then, PCR was performed using LA *Taq* polymerase (Takara). The primers used recognize the 500-bp fragment of TLR3 promoter containing E2F1 consensus sites. The primer sequences were 5'-CTCTCAGCTTTGCCATGTTTGGC-3' (sense) and 5'-CATTTTCATCAGGGAAGTGTGTGGC-3' (antisense). Primers for the CDC6 promoter and the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter (50) were used for positive and negative controls, respectively.

Immunoblotting. To analyze the protein expression of TLR3, E2F1, IκB-α, phosphorylated IκB-α, IRF-3, and phosphorylated IRF-3, we performed immunoblotting essentially according to our previously reported protocol (48). For Western blotting of Rb and p53, nuclear extracts were prepared from HCT116 cells according to a procedure we described previously (28). Protein samples were fractionated on an SDS-8% PAGE gel and transferred to polyvinylidene difluoride membrane. After blocking, the membranes were probed with the appropriate antibodies, and blots were visualized using SuperSignal (Pierce, Rockford, IL).

Statistical analysis. Data are presented as means ± the standard errors (SE) or standard deviations (SD), as indicated in the figure legends. The significance of the difference between the two groups was assessed using the Student unpaired two-tailed t test. For three or more group comparisons, we used one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test or with the Tukey-Kramer test (JMP software; SAS Institute). A P value of <0.05 was considered statistically significant.

RESULTS

Rb upregulates TLR3 mRNA and protein expression. To address the question of how Rb exerts its effect on antiviral response, we first analyzed the effect of Rb on TLR3 gene expression in MEF cells and in human epithelial cell line HCT116, which expresses endogenous functional TLR3 (48, 57). Interestingly, quantification of mRNA showed that the amount of TLR3 was lesser in Rb^{-/-} MEF compared to wild-type (WT) MEF (Fig. 1A). In HCT116 cells, TLR3 expression was also decreased by transfection of Rb siRNA (Fig. 1B). Conversely, TLR3 mRNA level was increased by Rb transfection in a dose-dependent manner (Fig. 1C). TLR3 protein expression was also upregulated by Rb overexpression (Fig. 1D) and decreased by Rb knockdown (Fig. 1E). In addition, we checked the effect of Rb on TLR3 expression in several human epithelial cell lines. We found that knockdown of Rb decreased the expression of TLR3 in these cells (Fig. 1F to H). The efficiency of Rb knockdown by siRNA in the human cell lines was determined (Fig. 1I to L). These results indicated that Rb increases TLR3 expression in human and mouse epithelial cells.

Rb modulates TLR3 signaling. Next, we assessed the effect of Rb on the TLR3 downstream cytokines IL-8/KC and IFN-β mRNA level using WT and Rb^{-/-} MEF stimulated with increasing

FIG 2 Rb modulates TLR3 signaling. (A to D) The mRNA level of cytokines (KC/IL-8 and IFN- β) were assessed by quantitative PCR in WT or Rb^{-/-} MEF (A and B) or in HCT116 cells (C and D) unstimulated or stimulated with the indicated concentration of poly(I:C) for 12 h (A and B) or 3 h (C and D). mRNA expression was normalized to mouse or human 18S rRNA. Values are means $\pm$ the SD of triplicate platings. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively, versus the control in WT MEF (A and B) or in si-GL2-transfected HCT116 cells (C and D). #, ##, and ### indicate $P < 0.05$, 0.01, and 0.001, respectively, versus the control in si-Rb-transfected HCT116 cells (C and D). P values were assessed by ANOVA with Dunnett's multiple comparison test. †, ††, and ††† indicate $P < 0.05$, 0.01, and 0.001, respectively, as assessed by t test. n.s., not significant. (E and F) The mRNA levels of the cytokines IL-8 (E) and IFN- β (F) were assessed by quantitative PCR in HCT116 cells transfected with si-GL2 (control), si-TLR3, or si-Rb and stimulated with 5 μ g of poly(I:C)/ml for 3 h. The mRNA level was normalized to 18S rRNA. Values are means $\pm$ the SD of triplicate platings. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively, versus the untreated control in each siRNA, as assessed by the Student t test. # and ### indicate $P < 0.05$ and 0.001, respectively, versus the poly(I:C)-treated si-GL2-transfected group, as determined by ANOVA with the Tukey-Kramer test. † and †† indicate $P < 0.05$ and 0.01, respectively, as assessed by the Student t test. (G and H) The ratio of the poly(I:C)-induced increase of cytokines (shown in panels E and F) in si-TLR3- or si-Rb-transfected cell relative to siGL2 was calculated.

dose of poly(I:C) for 12 h. In WT MEF, poly(I:C) dose dependently induced KC and IFN- β expression (Fig. 2A and B). However, in Rb^{-/-} MEF, the induction of cytokines after poly(I:C) stimulation was remarkably suppressed (Fig. 2A and B). In HCT116 cells, transfection of Rb siRNA also decreased the cytokine production in comparison to control siRNA after stimulation with poly(I:C) (Fig. 2C and D), albeit not to the same extent as that observed in Rb^{-/-} MEF. This may be due to the fact that unlike in Rb knockout MEF, knockdown of Rb by siRNA did not completely deplete Rb in HCT116 cells (Fig. 1E; Rb). Another possibility is that an Rb-independent mechanism(s) may be involved in

the slight response to poly(I:C) in si-Rb-transfected cells (48). Consistent with this idea, poly(I:C)-induced expression of IL-8 and IFN- β was slightly higher in cells transfected with si-Rb than with si-TLR3 (Fig. 2E to H). However, the blunted response to poly(I:C) in Rb^{-/-} MEF and Rb knockdown HCT116 cells compared to controls indicated that Rb affects TLR3 function such as cytokine induction in epithelial cells. To confirm that the effect of Rb on poly(I:C)-induced cytokine expression was via TLR3, we performed a rescue experiment. HCT116 cells were transfected with si-GL2 or si-Rb and cotransfected with pcDNA3.1 empty vector (con) or TLR3 expression plasmid. These cells were stimu-

FIG 3 Effect of Rb on poly(I:C)-induced cytokines is mediated by TLR3. (A and B) HCT116 cells were transfected with si-GL2 (black bars) or si-Rb (white bars) and cotransfected with 1 μ g of pcDNA3.1 (con) or TLR3 plasmid. At 48 h posttransfection, the cells were stimulated with 5 μ g of poly(I:C)/ml for 3 h, and the mRNA expression of IL-8 (A) and IFN- β (B) was determined by quantitative PCR. The mRNA level was normalized to 18S rRNA. Values are means $\pm$ the SD ($n = 3$). ** and *** indicate $P < 0.01$ and 0.001 , respectively, versus the respective unstimulated group, as assessed by the Student t test. # and ### indicate $P < 0.05$ and 0.001 , respectively, versus the pcDNA3.1 (con)/si-GL2-transfected, poly(I:C)-treated group, assessed by ANOVA with the Tukey-Kramer test. ††† indicates $P < 0.001$, assessed by the Student t test. (C and D) The ratio of the poly(I:C)-induced increase of cytokines (shown in panels A and B) relative to the pcDNA3.1/siGL2-transfected, poly(I:C)-treated group was calculated. (E) Cytoplasmic lysates from HCT116 cells transfected with 200 nM si-Rb or si-GL2 and unstimulated or stimulated with poly(I:C) for 3 h were analyzed by Western blotting with the indicated antibodies. Hsc70 was used as a loading control.

lated with poly(I:C), and the cytokine mRNA levels were assessed. Ectopic expression of TLR3 in cells depleted with Rb was able to compensate for the Rb-induced decrease of poly(I:C) response (Fig. 3A and B). The level of cytokine induction mediated by TLR3 in si-Rb-transfected cells was similar to that in control siRNA-transfected cells (Fig. 3C and D). These results suggest that Rb affects poly(I:C)-induced cytokine expression through TLR3. TLR3 controls cytokine expression via cellular signaling molecules I κ B and IRF3, wherein the phosphorylation of I κ B and IRF3 leads to IL-8 and IFN- β production, respectively (2). Knockdown of Rb by siRNA blocked the I κ B and IRF-3 phosphorylation after poly(I:C) treatment (Fig. 3E), indicating that Rb is critical for TLR3 signaling in response to poly(I:C) stimulation.

E2F1 decreases the expression of TLR3 in epithelial cells. We next aimed to clarify the mechanism of how Rb controls TLR3 expression. Because the tumor suppressor Rb regulates gene expression through the E2F transcription factor family (10, 38), we determined the effect of E2F1, a representative E2F transcription factor, on TLR3 expression. The exogenous addition of E2F1 in HCT116 cells reduced the TLR3 mRNA and protein expression (Fig. 4A and C). Conversely, si-E2F1 nucleotide transfection slightly upregulated the TLR3 mRNA and protein levels (Fig. 4B and D). In other human epithelial cell lines—A549, HepG2, and HeLa—the mRNA expression of TLR3 tended to increase upon E2F1 knockdown (Fig. 4E to G). These results indicated that E2F1, in contrast to Rb, negatively affects TLR3 expression. Intriguingly, overexpression of E2F1 in HCT116 cells suppressed not only TLR3 (Fig. 4A) but also the dsRNA-sensing receptors RIG-I and MDA5, whereas it activated protein kinase receptor PKR-1 (Fig. 4H to J), suggesting a role of E2F1 in viral sensing system modulation. We noted that although the overexpression of E2F1 signif-

icantly suppressed TLR3 level (Fig. 4A), the effect of si-E2F1 did not achieve statistical significance (Fig. 4B to G). This might be due to incomplete depletion of E2F1 by siRNA (Fig. 4K to N). A compensatory effect of other E2F family members could not also be ruled out (4). Several E2Fs can bind to a particular promoter and the loss of a single E2F generally revealed modest effects, suggesting redundancy in their functions (54, 56).

Rb and E2F1 differentially regulate TLR3 promoter activity. To establish the transcriptional effect of E2F1 on TLR3, we assessed the promoter activity of TLR3 in the presence of increasing amount of E2F1. Cotransfection of E2F1 plasmid with the TLR3 promoter (bp -2006/+48) decreased the reporter activity of TLR3 (Fig. 5A). On the other hand, increased levels of Rb caused a concomitant increase in TLR3 promoter activity (Fig. 5B). Next, to identify the region of TLR3 promoter that is important for its response to E2F1/Rb, we used three promoter constructs with various lengths, labeled “-2 kbp” (-2006/+48), “-1 kbp” (-987/+48), and “-0.5 kbp” (-504/+48). These constructs and E2F1 or Rb expression plasmid were cotransfected in HCT116 cells. Reporter assays revealed that the activity of all three constructs was downregulated by E2F1 but upregulated by Rb (Fig. 5C and D), suggesting that E2F1/Rb affected TLR3 transcriptional activity at least within the “-0.5 kbp” proximal region of TLR3 promoter. *In silico* analysis of TLR3 promoter showed three E2F consensus binding sites within the 0.5-kbp region upstream from the transcription start site (Fig. 5E). In addition, TLR3 promoter activity was increased by Rb, and decreased by E2F1, similarly in both HCT116 (wild-type) cells and HCT116 p53^{-/-} cells (7) (Fig. 5F and G); therefore, the effects of Rb and E2F1 on TLR3 promoter were independent of p53, which we previously reported to transactivate TLR3 (48).

FIG 4 E2F1 decreases the expression of TLR3 in epithelial cells. (A and B) HCT116 cells were transfected with 1.0 or 2.0 μ g of E2F1 expression plasmid (A) or with 50 nM si-E2F1 (B). Total RNA from these cells was isolated for analysis of TLR3 mRNA by quantitative PCR. (C and D) TLR3 protein was examined in HCT116 cells transfected with E2F1 plasmid (C) or with si-E2F1 (D). In the right panels, immunoblots were quantified by densitometry using Image Gauge software (v4.23; FujiFilm). TLR3 expression was normalized to Hsc70 (internal control), and the ratio of normalized TLR3 expression was computed relative to control. The data are mean $\pm$ the SE from three independent experiments. *, $P < 0.05$ (assessed by ANOVA with Dunnett's test). (E to N) The mRNA level of the indicated genes were examined by quantitative PCR in several epithelial cell lines or in HCT116 cells (H to K) transfected with 50 nM si-E2F1 or si-GL2 (con) or with 1.0 μ g of E2F1 expression plasmid or pcDNA3.1 vector (con). The mRNA values were normalized with 18S rRNA. Values are means $\pm$ the SD of triplicate platings. P values were assessed by ANOVA with Dunnett's test (A) or assessed by Student t test (B and E to N). **, $P < 0.01$; ***, $P < 0.001$ (versus the control). n.s., not significant.

Binding of E2F1 to TLR3 promoter is modulated by Rb. To confirm the importance of the E2F binding sites in TLR3 promoter, we created point mutants at these sites and transfected the wild-type (WT) or mutated (MT) promoter construct together with E2F1 or Rb expression plasmid in HCT116 cells. As shown in Fig. 6A, while the WT TLR3 promoter was responsive to the suppressive effect of E2F1, mutant promoter activity was not reduced by E2F1. Similarly, WT TLR3 promoter activity was increased by Rb, but the response to Rb was blunted in MT TLR3 promoter (Fig. 6B). The MT promoter had a slightly higher basal activity compared to WT probably due to the release from the inhibitory effect of endogenous E2F1 (Fig. 6A and B). To investigate whether E2F1 directly binds to the TLR3 promoter, a ChIP assay was performed using primers that recognize the "–0.5 kbp" TLR3 promoter region. Immunoprecipitation with antibody specific to E2F1 but not with control rabbit IgG antibody, and subsequent PCR revealed the endogenous association of E2F1 with the promoter region of TLR3 (Fig. 6C). Congruently, overexpression of E2F1 in HCT116 cells also showed the binding of exogenous E2F1 to TLR3 promoter (Fig. 6D). In these experiments, the binding of E2F1 to the promoter of CDC6, a known E2F1 target gene (24, 40), was performed to serve as a positive control, whereas the GAPDH promoter was used as a negative control (Fig. 6C and D). Collec-

tively, these results suggest that E2F1 binds to the TLR3 proximal promoter and decreases the expression of TLR3. In addition to confirm the correlation between Rb and E2F1 on TLR3 transcriptional regulation, we transfected the TLR3 promoter (–0.5 kbp) together with empty vector or Rb and/or E2F1. E2F1 alone drastically reduced the promoter activity of TLR3. Interestingly, the suppressive effect of E2F1 was titrated away by increasing amounts of cotransfected Rb plasmid (Fig. 6E). Conversely, the Rb-induced upregulation of TLR3 promoter activity was dose dependently inhibited by the cotransfection of E2F1 (Fig. 6F). Moreover, Rb suppressed the binding of E2F1 to TLR3 promoter (Fig. 6G). To determine whether the suppressive effect of E2F1 on TLR3 *per se* is independent of Rb, E2F1 was overexpressed in cells cotransfected with si-Rb. Interestingly, E2F1 was able to downregulate TLR3 mRNA level in si-Rb-transfected cells (Fig. 6H). Taken together, these results indicate that E2F1 can inhibit TLR3 transcription independently of Rb and that Rb regulates TLR3 by interfering with E2F1 binding to TLR3 proximal promoter.

Poly(I-C) increases Rb expression and suppresses E2F1 binding to TLR3 promoter. It was previously reported that various dsRNAs, including the synthetic dsRNA analogue poly(I-C), modulate the expression of p53 (29). However, whether poly(I-C) affects Rb is unclear. Thus, we checked Rb and E2F1 expression

FIG 5 Rb and E2F1 differentially regulate TLR3 promoter activity. (A and B) HCT116 cells were transiently transfected with TLR3 ($-2006/+48$ bp) promoter construct or pGL3b vector ($0.2 \mu\text{g}$) and 0.05 or $0.1 \mu\text{g}$ of E2F1 (A) or Rb (B) expression plasmid or empty vector. (C and D) HCT116 cells were transiently transfected with the indicated TLR3 promoter constructs ($0.2 \mu\text{g}$) and $0.1 \mu\text{g}$ of E2F1 (C) or Rb (D) plasmid or empty vector (con). For panels A to D, the luciferase activity was assayed 48 h posttransfection and is expressed as the fold activation over the pGL3b vector (A and B) or empty vector (C and D). Values are the mean $\pm$ the SE of triplicate platings. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus TLR3p, as assessed by ANOVA with Dunnett's test (A and B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control of each promoter construct, as assessed by using Student t test (C and D). (E) Schematic diagram of the -0.5 kb TLR3 promoter with the indicated positions of E2F binding site. Transcription start site is denoted with a $+1$. (F and G) HCT116 wild-type and HCT116 p53 $^{-/-}$ cells were transiently transfected with various lengths of TLR3 promoter construct and Rb (F) or E2F1 plasmid (G) or empty vector. The luciferase activity was assayed at 48 h posttransfection and is expressed as the fold activation over the control vector. Values are means $\pm$ the SE ($n = 3$). * and ** indicate $P < 0.05$ and 0.01 , respectively, against the control of each promoter construct in HCT116 p53 $^{+/+}$ cells. #, ##, and ### indicate $P < 0.05$, 0.01 , and 0.001 , respectively, against the control of each promoter construct in HCT116 p53 $^{-/-}$ cells. P values were assessed by Student t test.

after poly(I:C) treatment to clarify the molecular link between Rb and poly(I:C). A time course analysis indicated that poly(I:C) increased the Rb protein level at 6 and 12 h of treatment but did not affect E2F1 in HCT116 cells (Fig. 7A). The expression levels of Rb and E2F1 mRNA were not affected by poly(I:C) treatment (data not shown). On the other hand, p53 was decreased by poly(I:C) treatment (Fig. 7A), a finding consistent with the findings of Marques et al. (29). Concomitant with the poly(I:C)-induced increase of Rb expression, E2F1 binding to TLR3 promoter was suppressed, as determined by ChIP analysis (Fig. 7B). The poly(I:C)-induced loss of E2F1 binding to TLR3 promoter was prevented in si-Rb-transfected cells (Fig. 7C). Consistent with these results, the TLR3 mRNA level and promoter activity were increased by poly(I:C) treatment (Fig. 7D and E). Rb knockdown by siRNA in HCT116 cells diminished the poly(I:C)-induced expression of TLR3 compared to the si-control (Fig. 7F). Moreover, poly(I:C)-induced TLR3 expression was clearly suppressed in Rb $^{-/-}$ MEF cells compared to WT MEF (Fig. 7G). These results demonstrated

that Rb is important for the induction of TLR3 expression in response to poly(I:C).

DISCUSSION

One of the important ways in which tumor suppressor proteins inhibit tumorigenesis caused by viral oncogenes is by influencing the antiviral host defense system (21, 44, 47). Indeed, a deficiency in the expression of a tumor suppressor protein, such as Rb or p53, increases viral proliferation and decreases host survival (35, 36, 47). Conversely, viruses exert their tumorigenic effect by dampening the tumor suppressor proteins' activities that could result in the repression of antiviral host protection system (29, 34). In the present study we show that Rb positively influences the expression of the viral sensing receptor TLR3 and newly identifies Rb as an important protein for antiviral host protection. This adds to the accumulating reports that shed light on how tumor suppressor proteins affect antiviral host response. Combined with our previous report (48), this further proves that the tumor suppressor gene

FIG 6 Rb regulates TLR3 promoter activity by interfering with the binding of E2F1 to TLR3 promoter. (A and B) HCT116 cells were transfected with 0.2 μ g of pGL3b vector or 0.5-kbp TLR3 wild-type (WT) or E2F-site mutant (MT) promoter and cotransfected with 0.1 μ g of E2F1 (A) or Rb (B) expression plasmid or pcDNA3.1 empty vector (con). The luciferase activity was determined 48 h after transfection and is expressed as the fold activation over the pGL3b vector. Values are mean $\pm$ the SE ($n = 3$). †††, $P < 0.001$ versus pGL3b; *, $P < 0.05$, and ***, $P < 0.001$ versus WT TLR3 promoter/pcDNA3.1 (con), as assessed by ANOVA with the Tukey-Kramer test. n.s., not significant. (C and D) E2F1 binding on TLR3 promoter was determined by ChIP assay using nuclear extracts of HCT116 cells (C) or of HCT116 cells transiently transfected with E2F1 plasmid (D). Immunoprecipitation was done using antibody specific for E2F1 or with rabbit IgG as a control. Primers recognizing TLR3 (−504/+48 bp), CDC6, and GAPDH promoters were used for PCR. CDC6 promoter was used as a positive control, and GAPDH promoter was used as a negative control for E2F1 binding. (E and F) HCT116 cells were transiently transfected with the “−0.5 kbp” TLR3 promoter construct or pGL3b vector (0.2 μ g), 0.1 μ g of E2F1 (E) or Rb (F), and the indicated amount of E2F1 or Rb plasmid or empty vector. The luciferase activity was determined 48 h after transfection and is expressed as the fold activation over the pGL3b vector. Values are the means $\pm$ the SE of triplicate platings. †††, $P < 0.001$ versus pGL3b; ###, $P < 0.001$ versus TLR3p, determined by a Student t test; *, $P < 0.05$, and ***, $P < 0.001$ versus E2F1 (E) or Rb (F), assessed by ANOVA with the Dunnett's test. (G) E2F1 binding on the TLR3 promoter was determined by ChIP assay using nuclear extracts of HCT116 cells transfected with E2F1 and/or Rb. (H) HCT116 cells were transfected with 50 nM si-GL2 or si-Rb and cotransfected with pcDNA3.1 (con) or E2F1 expression plasmid (1.0 μ g). At 48 h posttransfection, the total RNA was isolated, and the TLR3 mRNA level was analyzed by quantitative PCR. mRNA values were normalized with 18S rRNA. Values are means $\pm$ the SD of triplicate platings. * and **, $P < 0.05$ and 0.01, respectively, versus con/siGL2, assessed by ANOVA with the Tukey-Kramer test.

network deeply correlates with host protection against viral infection not only by regulating apoptosis but also by inducing an antiviral immune molecule. Rb increases TLR3 expression at the transcriptional level via its regulation of the transcription factor E2F1. Rb also positively regulates cytokine expression, a finding consistent with a previous report (21). In addition, we found that Rb is critical for TLR3 induction after poly(I:C) stimulation, a capacity that is distinct from p53, which we also previously demonstrated to transcriptionally activate basal TLR3 (48). Whereas poly(I:C) transiently downregulates p53, it upregulates Rb expression and subsequently inhibits E2F1 binding to the TLR3 promoter resulting in increased TLR3 promoter activity and expression level. Rb knockdown suppressed TLR3 induction in poly(I:C)-stimulated cells. Together, these data imply that p53 and, in part, Rb may regulate the basal expression of TLR3 and that Rb, through E2F1, critically affects the inducible TLR3 expression level.

E2F1 is known to usually transactivate its target genes such as cyclin E and CDC6 (39, 40). However, several reports indicated that E2F1 not only activates but also may inhibit its target gene by directly binding to its promoter region (12, 13, 31, 55). In fact, by genome-wide expression analysis, an unexpectedly large number of genes have been found to be transcriptionally downregulated by overexpression of E2F1 directly and independently of Rb (33, 52, 60). In the present study, we showed that the TLR3 gene is a novel

target gene that is suppressed by E2F1 by directly binding to TLR3 gene promoter. Because E2F1 repressed TLR3 at a relatively similar level in both si-Rb- and control siRNA-transfected cells (Fig. 6H), then E2F1 could exert its suppressive effect independently of Rb. However, when Rb was activated by poly(I:C), it sequestered E2F1 away from TLR3 promoter, relieving the E2F1's suppressive effect on its target gene (Fig. 7). This result is akin to the findings of Muller et al., who showed that the genes suppressed by E2F1 (*PAI1* and *CTGF*) were increased by overexpression of Rb, and these researchers hypothesized that active Rb binds to and inactivates E2F1 (33). Similarly, it was previously shown by Koziczak et al. that plasminogen activator inhibitor 1 (*PAI-1*) is repressed by E2F1 independently of pocket proteins Rb and p107/p130 and that the *PAI-1* gene was derepressed by the presence of Rb, which sequestered E2F1 away from *PAI-1* promoter (26, 27). How E2F1 suppresses transcription is unknown. E2F1, E2F2, and E2F3a are often considered as activators, while E2F3b, E2F4, and E2F5 are often referred to as repressors (10, 15). However, the transactivation domain and the Rb binding domain in these E2F members overlap; thus, E2Fs 1 to 5 may potentially function as both activators and repressors (4, 14). The assembly of proteins at a particular promoter and the specific cellular environment may determine whether an E2F family member activates or represses transcription (20, 56). It was proposed that the E2F “activators” E2F1 to E2F3 might also be able to recruit corepressors to block transcrip-

FIG 7 Poly(I-C) increased Rb expression and suppressed E2F1 binding to TLR3 promoter to induce the expression of TLR3. (A to E) HCT116 cells were unstimulated or stimulated with 5 μ g of poly(I-C)/ml for the indicated times or for 12 h (C). Cytoplasmic lysates or nuclear extracts were analyzed by immunoblotting with the indicated antibodies. Tubulin and Hsc70 were used as loading controls for nuclear extracts and cytoplasmic lysates, respectively (A). E2F1 binding to TLR3 promoter (bp -504/+48) was analyzed by ChIP assay using nuclear extracts of poly(I-C)-treated HCT116 cells (B) or nuclear extracts of cells transfected with si-GL2 or si-Rb and unstimulated or stimulated with poly(I-C) (C). TLR3 mRNA level (D) and TLR3 promoter activity (E) were determined in poly(I-C)-treated HCT116 cells. (F) TLR3 mRNA level was analyzed in HCT116 cells transfected with si-Rb or si-GL2 and stimulated with 5 μ g of poly(I-C)/ml for 12 h. (G) WT and Rb^{-/-} MEF were stimulated with 5 μ g of poly(I-C)/ml for the indicated time, and TLR3 mRNA level was assessed by quantitative PCR. For panel E, the luciferase activity is expressed as the fold activation over the control (0 h). Values are means $\pm$ the SE of triplicate platings. For panels D, F, and G, the mRNA values were normalized to 18S rRNA. Values are means $\pm$ the SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the control (0 h), as assessed by ANOVA with the Dunnett's test (D and G). n.s., $P > 0.05$ versus unstimulated si-GL2- and si-Rb-transfected cells, respectively, as assessed by the Student t test (F).

tion in a manner similar to the way MYC suppresses the expression of some of its target genes (6). Congruently, binding of E2F1 could induce conformational changes in the chromatin structure of the target promoter, as shown by Koziczak et al. for the PAI-1 gene (27).

Whether other E2Fs affect TLR3 expression remains a future topic to be explored, but we have shown here that Rb governs TLR3 ligand response through the disruption of E2F1 binding to TLR3 promoter (Fig. 7B and C). TLR3 is an inducible molecule whose expression in normal physiological state (i.e., absence of infection) is kept relatively low (3). The endogenous binding of E2F1 to TLR3 promoter implies that E2F1 may constitutively modulate the basal expression of TLR3, a regulation that is necessary to prevent unwarranted activation of TLR3. Aside from TLR3, host cells have several dsRNA-sensing molecules, i.e., PKR, RIG-I, and MDA5, which are also important for viral recognition (2). Thus, we checked the effect of E2F1 on the expression of these molecules. We found that E2F1 also decreased the mRNA level of dsRNA receptors RIG-I and MDA5, but it transactivated the dsRNA-dependent protein kinase PKR (Fig. 4H to J). Although it has been reported that E2F1 transactivates PKR by directly binding to PKR promoter (53), our report is the first to show that E2F1 suppresses MDA5 and RIG-I. However, whether E2F1 directly regulates these RNA helicases and whether their regulation involves Rb remains unclear. The suppression exerted by E2F1 on the viral recognition receptor TLR3 (also MDA5 and RIG-I) is noteworthy

because this may be an integral part of how E2F-Rb pathway modulates the viral sensing system for host protection.

ACKNOWLEDGMENTS

The HCT116 p53^{+/+} and HCT116 p53^{-/-} cells were generously provided by Bert Vogelstein. The pSG5-Rb plasmid was kindly provided by Mark Ewen.

This study is supported by Grants-in-Aid for Science Research from the Ministry of Education, Science, Sports, and Culture of Japan (MEXT) (22390015 to H.K., 23590082 to M.S., and 21107522 to S.O.); by the Global COE Programs (Cell Fate Regulation Research and Education Unit and Research Center Aiming at the Control of AIDS) from MEXT; by a Health and Labor Sciences Research Grant from the Ministry of Health, Labor, and Welfare of Japan (H22-AIDS-I-002 to S.O.); by the Japan Foundation for AIDS Prevention fellowship; and by a Sasagawa Scientific Research Grant from The Japan Science Society (to M.T.).

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