Interferon Regulatory Factor 5 Represses Expression of the Epstein-Barr Virus Oncoprotein LMP1: Braking of the IRF7/LMP1 Regulatory Circuit

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Received 13 April 2005/Accepted 6 June 2005

We have reported evidence for a positive regulatory circuit between interferon regulatory factor 7 (IRF7) and the Epstein-Barr virus (EBV) oncoprotein 1 (LMP1) (S. Ning, A. M. Hahn, and J. S. Pagano, *J. Virol.* 77:9359–9368, 2003). To explore a possible braking mechanism for this circuit, several type II EBV-infected cell lines that express different levels of LMP1 and IRF7 proteins and therefore are convenient for studying modulation of expression of LMP1 were analyzed. Endogenous levels of IRF7 and LMP1 were directly correlated. Transient expression of an IRF7 dominant-negative mutant decreased LMP1 levels. Endogenous IRF5 and IRF7 proteins were shown to physically associate in EBV-positive cells. Transient expression of IRF5 decreased activation of the LMP1 promoter by IRF7 in a dose-dependent manner. Finally, transfection of either an IRF5 dominant-negative construct or IRF5 small interfering RNA in these cells resulted in increases in endogenous levels of LMP1. These results indicate that IRF5 can downregulate IRF7's induction of expression of LMP1 most likely by interacting with IRF7 and provide a means of modulating a regulatory circuit between IRF7 and LMP1.

The interferon (IFN) regulatory factor (IRF) family, which consists of 10 members, plays essential roles in the activation of innate immune response to viral infection (reviewed in references 3, 7, 11, 12, and 24). IRF3 and IRF7, two of the family members, are indispensable for induction of alpha/beta IFNs (IFN- α/β) upon virus infection (reviewed in references 3, 7, 12, and 24) through Toll-like receptor (TLR) signaling (6, 8, 13, 14, 17, 24, 30, 33, 42). IRF7 is now considered the master regulator of IFN- α/β -dependent immune responses (15). IRF5, characterized more recently, also participates in induction of IFN- α/β upon virus infection (4) through TLR signaling (32, 37). Thus, three members of the IRF family, IRF3, IRF7, and IRF5, are directly involved in signaling pathways triggered by virus infection (reviewed in references 3 and 24).

As the versatile principal oncoprotein of the human gamma herpesvirus Epstein-Barr virus (EBV), latent membrane protein 1 (LMP1) has been extensively studied and is remarkable for its ability to activate at least seven signaling pathways, including the NF- κ B, p38/MAPK, Jak/STAT, JNK/AP-1, and PI3K/Akt pathways, via its C-terminal activation domains. The other two pathways, ubiquitination and CDC42 activation, are mediated by LMP1 N-terminal and transmembrane domains, respectively (reviewed in references 9 and 20). Induction of expression of LMP1 depends on several virus-specific factors, the best characterized of which is the transcriptional activator EBV nuclear antigen 2 (EBNA2) (16, 34, 35, 38, 40).

Our previous work uncovered the intimate involvement of IRF7 in EBV latency and showed first that IRF7, along with IRF2, represses the promoter used for expression of EBNA1

in type I latency in which latent gene expression is most restricted (45). We then detailed a special relationship between IRF7 and LMP1, which is capable of both inducing expression of and activating IRF7 (46, 49). Subsequently, we showed that there is also a reciprocal action of IRF7 on expression of LMP1 in that the cellular protein can activate the LMP1 promoter and induce expression of the viral protein (28). Most recently, we have demonstrated that expression of IRF7 is itself upregulated by IRF7 in an IFN-independent manner (29). Thus, expression of LMP1 and that of IRF7 are closely linked, which opens the possibility of functional consequences.

This linkage is reflected biologically in functional interactions of the viral and cellular proteins. Besides the established oncogenicity of LMP1, IRF7 itself has oncogenic properties, which enhance LMP1's oncogenicity in cell-culture assays and tumor formation in animal models (50). The most interesting verification of a possible physiological interaction of these two proteins comes from discovery of overexpression and activation of IRF7 in EBV-positive central nervous system lymphomas (50). Thus, not only does IRF7 induce LMP1, but it also appears to potentiate its oncogenic effects. Consequently, how modulation of the regulatory circuitry of these two proteins is brought about becomes an important question. Specifically, if IRF7 functions to upregulate LMP1, is there a mechanism for dampening or braking such an effect?

It has been reported that IRF5/IRF3 heterodimers and IRF5/IRF5 homodimers can activate IFN- α gene (IFNA) transcription, since in virus-infected cells transfected with IRF5 the levels of IFNA transcripts are higher in the presence than in the absence of IRF3. In the absence of both IRF3 and IRF7, IRF5 alone is able to bind to the virus-responsive element of IFNA genes and induce low levels of IFN- α in virus-infected cells (4). However, IRF5 also forms heterodimers with IRF7, and IRF5/IRF7 complexes repress IFNA transcription in virus-

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infected cells, since IFNA transcription could not be activated in cells cotransfected with IRF5 and IRF7, whereas it could be activated in cells transfected with either IRF5 or IRF7 alone (1). Thus, IRF5/IRF3 heterodimers and IRF5/IRF5 homodimers activate but IRF5/IRF7 heterodimers repress transcription of the same set of genes.

In this study, we show that endogenous IRF5 and IRF7 can physically associate and that IRF5/IRF7 heterodimers inhibit the induction by IRF7 of expression of LMP1 in EBV-infected cells. Reduction of endogenous levels or activity of IRF5 produces increased levels of LMP1.

MATERIALS AND METHODS

Cell lines. The EBV-infected human breast cancer cell line MDA-MB-231 clones C4A3, C1D12, C2G6, and C3B4 were generous gifts from Irene Joab (INSERM EPI 03-34, IUH, Hospital Saint-Louis, Paris, France) and were maintained in RPMI 1640 medium supplied with 2 mM L-glutamine, 700 µg/ml G-418 (GIBCO), 10% fetal bovine serum (FBS), and penicillin-streptomycin.

BJAB is an EBV-negative human Burkitt's lymphoma (BL) cell line. JiJoye is a type III EBV-positive human BL cell line. P3HR-1, a mutant cell line derived from JiJoye, has a deletion of portions of the EBNA-LP and EBNA2 open reading frames and expresses low levels of LMP1. All the cell lines were grown in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin. The 293 cell line, derived from human kidney epithelial cells, was grown in Dulbecco's modified Eagle medium with 10% FBS and penicillin-streptomycin.

Plasmids and antibodies. pcDNA3-IRF7A (45), pCMVSPORT-IRF5 (4), and Flag-IRF5 (4) were described previously. Flag-IRF5DN was constructed by inserting IRF5DN (deletion of DNA-binding domain, amino acids 35 to 136) into pCMV2-Flag vector. pGL2(-512/+72)LMP1p-Luc has two directly repeated copies of the LMP1 -512/+72 promoter element and was a gift from Jeffery Lin and Elliott Kieff (21). The pGL3/IFN-βp-Luc construct was described previously (22).

IRF7 rabbit polyclonal antibody (H-246) was purchased from Santa Cruz, IRF5 goat polyclonal antibody (ab2932) was purchased from Abcam, EBNA2 (PE2) and LMP1 (CS1-4) monoclonal antibodies were from Dako, and γ-tubulin monoclonal antibody was from Sigma.

IRF5 RNA interference (RNAi) assay. Cells in 60-mm dishes were transfected with 60 nmol IRF5 small interfering RNA (siRNA; Ambion) or negative control siRNA (Ambion) with the use of TransPass R1 transfection reagent (New England BioLabs Inc.) following the manufacturer's instructions. The negative control siRNAs are comprised of a 19-bp scrambled sequence with 3' dT overhangs; the sequences have no significant homology to any known gene sequences from mice, rats, or humans. The IRF5 siRNA targets both transcript variants of human IRF5, NM_002200 exon 2 and NM_032643 exon 3. The medium was replaced 12 h after transfection. Cells were collected for Western blot analyses after 60 h.

Transfection and reporter assays. 293 cells in 12-well plates were transfected with an Effectene kit following the manufacturer's instructions (QIAGEN). For 12-well plates, 0.4 µg total plasmids was transfected. Vector DNA was added to equalize the total amount of DNA used in all transfections. Cells were collected 48 h after transfection. For luciferase assays, the transfected cells were collected 48 h posttransfection. Cell lysates (20 µl each) were combined with luciferase assay reagent (Promega), and the relative light units were measured in an Lmax luminometer (Molecular Devices Corp.). The transfection efficiencies were normalized to β-galactosidase values. Reporter assay results presented are from single experiments representative of multiple independent trials.

P3HR1 cells were transfected with 5 µg plasmids with an electroporator at 210 V and 975 µF. After electroporation, cells were resuspended in 10 ml complete medium and incubated for 48 h.

Immunoprecipitation. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Proteins (200 µg) in cell lysates were applied for each immunoprecipitation. Cell lysates (500 µl) were precleared with 1 µg of appropriate immunoglobulin G (IgG) plus 50 µl of protein A/G beads (Santa Cruz) for 1 h. After preclearing, cell lysates were incubated with 1 µg of antibody or IgG for 4 h. Beads were extensively washed with RIPA buffer and resuspended in an equal volume of 2× SDS loading buffer and RIPA buffer.

Western blotting. Cells were lysed in RIPA lysis buffer as above. Cells lysates (100 µg of total proteins each lane) were separated on 10% SDS-polyacrylamide gel electrophoresis gels for detection of IRF7, IRF5, and LMP1. The proteins

FIG. 1. Correlation of IRF7 with LMP1 protein levels in type II EBV-positive cells. (A) Cell lysates prepared from C1D12, C2G6, C3B4, and C4A3 were subjected to Western blot analysis with specific antibodies to LMP1 and IRF7. For each lane, 150 µg of total protein was loaded. (B) C3B4 cells, which express the highest levels of LMP1, were transfected with Flag-IRF7DN or empty vector pCMV2-Flag. After 48 h, cell lysates were prepared and Western blotting was performed with LMP1 and IRF7 antibodies.

were then transferred to nitrocellulose membranes. Membranes were blocked in 5% milk before incubation with specific antibodies for 2 h at room temperature. The specific primary antibodies were applied at dilutions of 1:500 for IRF7 and IRF5, 1:300 for LMP1, and 1:10,000 for γ-tubulin. Appropriate horseradish peroxidase-conjugated secondary antibodies were applied at a dilution of 1:3,000 for 1 h at room temperature. Specific signals were detected by enhanced chemiluminescence following the manufacturer's protocol (Amersham Pharmacia Biotech).

RESULTS

IRF7 and LMP1 levels directly correlate in type II EBV-positive cells. In a previous study we showed that when IRF7 is introduced into P3HR1 cells, which have low levels of IRF7 and lack EBNA2, it can induce LMP1 expression independently of EBNA2. This finding offered an explanation for how LMP1 might be expressed in type II cells where EBNA2 protein is absent (28). To test this idea further, but directly in type II cells, we examined endogenous IRF7 and LMP1 levels in a series of cell lines, the C1D12, C2G6, C3B4, and C4A3 clones, all of which are EBV-infected cells with the type II phenotype derived from the human breast cancer cell line MDA-MB-231. These clones express different levels of LMP1 and do not express EBNA2. Western blotting results show consistent correlation of IRF7 and LMP1 levels in these clones (Fig. 1A). C3B4 cells have the highest level of both LMP1 and IRF7, whereas in C4A3 cells LMP1 is not detectable and the IRF7 level is low. These results are consistent with findings in a previous study (39). Further, we transfected the clone with the highest level of IRF7, C3B4, with an IRF7 dominant-negative construct and showed that reduction in IRF7 activity decreased

FIG. 2. Association of IRF7 with IRF5 in EBV-positive cells. (A) Detection of endogenous IRF5 in different cell lines. For each lane, 100 μ g of total protein was loaded. (B) IRF5 is associated with IRF7 in EBV-positive cells. Total protein (200 μ g) in cell lysates was precleared with normal IgG and divided into equal amounts. One was subjected to immunoprecipitation (IP) with specific antibodies against IRF5 or IRF7, and the other with normal IgG. After extensive washing, immunoprecipitates were analyzed by Western blotting with IRF5 or IRF7 antibodies. IB, immunoblot.

the level of LMP1 (Fig. 1B). Thus, the LMP1 expressed in these type II cells appears to depend on expression of IRF7.

IRF5 interacts with IRF7 in EBV-positive cells. Expression of IRF7 induces the LMP1 promoter and increases levels of LMP1 RNA and proteins (28). To explore whether IRF5 can interact with IRF7 and affect its ability to induce expression of LMP1, first we checked the endogenous IRF7 and IRF5 levels in a variety of cell lines, including EBV-negative BJAB cells and type II and type III cells. As shown in Fig. 2A, in all the cell lines tested both IRF7 and IRF5 were detected. Levels of IRF7 were lower in the breast cancer cells, which is expected because IRF7 is preferentially expressed in cells of lymphoid origin (45). Human IRF5 protein has two isoforms resulting from two transcripts of 2.4 and 3.4 kilobases (4), and the IRF5 antibody used in this study recognizes both isoforms. Next, we performed immunoprecipitation assays in three representative EBV-positive cell lines, including the type II C3B4 cells and type III P3HR1 and JiJaye cells. In the cell lysates immunoprecipitated with IRF7 antibody, IRF5 was detected in all cases (Fig. 2B, upper panel). In the converse experiment, in immunoprecipitates of the cell lysates incubated with IRF5 antibody, IRF7 was detected in all three cell lines (Fig. 2B, lower panel). Thus, the results show that endogenous IRF5 physically interacts with IRF7 in EBV-infected cells.

Transient expression of IRF5 reduces LMP1 promoter activity induced by IRF7. To test whether IRF5 might affect the function of IRF7 we studied its effect on induction of the LMP1 promoter by IRF7. 293 cells were cotransfected with an

FIG. 3. IRF5 represses IRF7 activation of the LMP1 promoter. 293 cells in 12-well plates were cotransfected with pLMP1p-Luc, IRF7, and increasing amounts of IRF5 expression plasmid. β -Galactosidase expression plasmid was cotransfected as an internal control. Equal amounts of cell lysates were assayed for luciferase activity 48 h after transfection. Activation is shown relative (*n*-fold) to the basal level of the reporter gene response to empty vector.

LMP1 promoter-luciferase reporter construct, pLMP1p-Luc, a constant amount of an IRF7 expression construct, pcDNA3-IRF7, and increasing amounts of an IRF5 expression construct, pcDNA3-IRF5. As expected, IRF7 alone activated pLMP1p-Luc 10-fold. IRF5 alone had little if any effect on the LMP1 promoter. However, expression of increasing amounts of IRF5 progressively decreased LMP1 promoter activity induced by IRF7 in a dose-dependent manner (Fig. 3). Thus, expression of IRF5 could override the transactivation function of IRF7.

Inhibition of IRF5 function or repression of IRF5 expression elevates LMP1 levels in type II EBV-positive cells. Since the C1D12 and C2G6 clones express very low levels of LMP1, we used these two cell lines in IRF5 dominant-negative and RNAi assays to check if endogenous LMP1 levels can increase after inhibition of IRF5 function or repression of its expression. First, we transfected these two lines and the B-cell line P3HR1 with an IRF5 dominant-negative construct and determined changes in LMP1 levels. As shown in Fig. 4A, expression of IRF5DN consistently increased LMP1 protein levels. Interestingly, in P3HR1 cells, where there was no detectable LMP1, a low level of LMP1 could be detected after expression of IRF5DN. No differences in IRF7 levels were detected in cells transfected with empty vector and IRF5DN (Fig. 4A). Thus, the increases in endogenous levels of LMP1 resulted from decreased levels or function of IRF5, not from changes in IRF7 levels. Inhibition of IRF5 function by IRF5DN is tested (Fig. 4B).

Next, we used RNAi to IRF5 to reduce its expression levels in type II cells. IRF5 siRNA (siIRF5) and scrambled negative-control siRNA oligomers were transfected into C1D12 and C2G6 cells. Western blot analyses for LMP1, IRF5, and IRF7 were performed after 60 h. As shown in Fig. 4C, in cells transfected with negative-control siRNA, levels of LMP1 were the same as in mock-transfected cells. However, there were significant elevations of LMP1 protein levels detected in cells

FIG. 4. Repression of IRF5 function increases LMP1 protein levels in type II cells. (A) Overexpression of an IRF5 dominant-negative mutant elevates LMP1 levels in type II C1D12 and C2G6 cells and in type III P3HR1 cells. C1D12 and C2G6 cells in 60-mm dishes were transfected with 2 μ g pCMV2-Flag empty vector or pCMV2-Flag/IRF5DN with the use of an Effectene kit. P3HR1 cells were transfected with 5 μ g of plasmids by electroporation, as described in Materials and Methods. Cells were collected 48 h after transfection, and cell lysates were used for Western blot analyses with specific antibodies against LMP1 and IRF7. (B) Inhibition of IRF5 function by the tested IRF5DN. A pGL3/IFN- β -Luc construct was used. A promoter-reporter assay was performed as above. (C) Silencing of expression of endogenous IRF5 by siIRF5 elevates LMP1 levels. C1D12 and C2G6 cells in 60-mm dishes were transfected with 60 nmol siIRF5 or negative control siRNA. Medium was changed after 12 h. Cells were collected 60 h after transfection, and lysates were used for Western blot analyses with specific antibodies against LMP1, IRF5, and IRF7.

transfected with siIRF5, and, correspondingly, the levels of IRF5 protein were obviously decreased, but IRF7 levels were again unchanged (Fig. 4C). The IRF5 RNAi results indicate that repression of IRF5 expression could increase endogenous LMP1 levels in type II cells. These results are consistent with the idea that an upregulatory circuit between IRF7 and LMP1 can be retarded by IRF5.

DISCUSSION

A function for IRF5 was revealed only recently when it was shown to induce expression of distinct IFNA gene subtypes upon Newcastle disease virus, vesicular stomatitis virus, and herpes simplex virus type 1 infection (2, 4). Appreciated more recently is the ability of IRF5, as well as IRF7, to stimulate a broad profile of host genes encoding other proteins in addition

to antiviral proteins (5). In contrast to its ability to activate IFNA promoters (4), which is shared by IRF7 (reviewed in references 7, 19, and 47), IRF5 has no detectable effect on the LMP1 promoter (Fig. 3), whereas IRF7 can induce expression of LMP1 as shown previously (28) and confirmed here, although it has a lesser effect than EBNA2 (Fig. 5, pathway 1). Instead, IRF5 inhibits expression of LMP1 in type II cells, most likely by interacting with IRF7. These findings implicate IRF5 in the modulation of stimulatory effects produced by IRF7 on expression of LMP1. This modulatory mechanism, which uses different signaling pathways from EBNA2, the prime enhancer used for LMP1 expression in type III cells (16, 34, 35, 38, 40, 44a), becomes evident only in the absence of expression of EBNA2, the more potent activator.

Regulation of LMP1 transcription is critical because it is

FIG. 5. A scheme for type II latency modulation. → indicates positive regulatory pathways, and ⊥ indicates a negative pathway. ① LMP1 induces expression of IRF7 through an unknown mechanism, probably through the NFκB pathway (46). ② LMP1 also promotes phosphorylation and nuclear translocation of IRF7 (48). ③ IRF7 can induce expression of LMP1 by binding to the IFN-stimulated response element (ISRE) of the LMP1 promoter, as shown previously (28) and confirmed here. ④ IRF7 is capable of positive autoregulation by binding IRF7-containing virus-activated factor to the ISRE and IRF-binding element in the IRF7 promoter and first intron (29). ⑤ In this study, we show that endogenous IRF5 and IRF7 associate and IRF5/IRF7 negatively regulates the positive circuit between IRF7 and LMP1.

essential for EBV's hallmark function, growth transformation of B lymphocytes (21, 35, 36). The finding that IRF7 induces expression of LMP1 provides a possible partial explanation for how LMP1 is expressed, although at low levels, in type II cells in the absence of EBNA2 (28) (Fig. 5, pathway 1). We have also shown that LMP1 can induce expression of IRF7 in addition to promote IRF7 phosphorylation (46) (Fig. 5, pathways 2 and 3), which may help to explain why IRF7 is expressed at appreciable levels in type II nasopharyngeal carcinoma tissues (L. Zhang and J. Pagano, unpublished results). The present results confirm that there is a positive regulatory circuit between IRF7 and LMP1, which as previously reported potentiates both expression and oncogenic properties of the two genes (28, 50). Recently, we also demonstrated that IRF7 can be autoregulated by binding IRF7-containing virus-activated factor to the IRF7 promoter (29), thereby promoting the circuit (Fig. 5, pathway 4). The main results in this paper now show that endogenous IRF5 and IRF7 can associate in EBV-infected cells and suggest that IRF5/IRF7 heterodimers negatively regulate this circuit (Fig. 5, pathway 5) so that a possible "runaway" effect on expression of the toxic LMP1 oncoprotein (10, 27) is modulated. Also, our findings add new elements as to how type II latency is maintained.

Certain members of the IRF family have an IRF association domain, which confers on each of them the ability to associate with itself or another IRF member in activating their target genes (25). For example, IRF8 interacts with IRF1 or IRF2 (reviewed in reference 18), and IRF3 and IRF7 can form IRF3/IRF3 and IRF7/IRF7 homodimers and IRF3/IRF7 heterodimers in induction of IFN-α/β (22, 29, 41, 43, 44). As to IRF5, it can form IRF5/IRF5 homodimers and IRF5/IRF7 and

IRF5/IRF3 heterodimers. IRF5/IRF3 and IRF5/IRF5 can activate IFNA gene transcription, whereas IRF5/IRF7 inhibits it (1, 2, 4). Thus, IRF5 may function either to inhibit or to stimulate expression of IFNA genes, depending on its binding partners. This inhibition results from masking of the IRF5 and IRF7 DNA-binding domains after heterodimer formation (1). Definitely, masking of the IRF5 and IRF7 DNA-binding domains abolishes the ability of these proteins to bind to all their targets, including LMP1. Thus, masking also provides an explanation for the inhibition of induction of LMP1 by IRF5/IRF7 interaction as shown in this study, and it is likely that the effect of IRF5 on IRF7 is not restricted to IFNA and LMP1 genes. We hypothesize that the balance between supply of IRF5 and IRF7 protein molecules may determine if the positive circuit between IRF7 and LMP1 becomes negatively regulated.

Perhaps the most striking findings in this study are provided by the effects of the IRF5 dominant-negative construct and by IRF5 RNAi in EBV-infected cells. Both repression of the function of IRF5 and reduction of levels of this protein produced increases in levels of endogenous LMP1 protein (Fig. 4). In these different cell lines, since the endogenous levels of IRF7 were unchanged in all cases, these results suggest a direct mechanism for modulation of LMP1 levels in type II cells.

Although we only show evidence of negative modulation of LMP1 by IRF5, we believe that other IRF family members may be involved in regulation of this circuit by induction or inhibition of expression of IRF7 and LMP1. IRF1, which can bind to the IRF7 promoter, is suggested to activate the IRF7 promoter in IFN-treated cells (23), and IRF9 can induce IRF7 upon virus infection and IFN treatment (23, 26, 31). In fact, IRF2, which usually functions as a transcriptional repressor, is also capable of binding to the IRF7 promoter strongly, whereas IRF5 has a very weak ability to bind to the IRF7 promoter, as shown by *in vitro* binding assays (data not shown). Thus, a complex rigorously regulatory network that controls expression of LMP1, governed by interplay between viral and cellular factors, is emerging. These interactions, exemplified by the type II latent infection state, are likely to have significance for EBV latency, oncogenesis, and evasion of host defense responses.

ACKNOWLEDGMENTS

We thank Irene Joab for the MDA-MB-231 EBV-infected clones, Betsy J. Barnes and Paula M. Pitha for IRF5 constructs, J. Lin and E. Kieff for pGL2(-512/+72)LMP1p-Luc plasmid, and R. Lin and J. Hiscott for the pGL3/IFN-βp-Luc plasmid. We also thank Angela M. Hahn for discussion of the work.

This research was supported by grants from the National Institute of Allergy and Infectious Diseases (AI 42372-01) and from the National Cancer Institute (CA 19014). L. Huye was supported by an NCI training grant (T32-CA09156-27).

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