Inactivation of Interferon Regulatory Factor-1 Tumor Suppressor Protein by HPV E7 Oncoprotein

IMPLICATION FOR THE E7-MEDIATED IMMUNE EVASION MECHANISM IN CERVICAL CARCINOGENESIS*

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The abbreviations used are: HDAC, histone deacetylase; IRF, interferon regulatory factor; IFN, interferon; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; STAT, signal transducers and activators of transcription, TSA, trichostatin.

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In studying biological roles of interferon regulatory factor (IRF)-1 tumor suppressor in cervical carcinogenesis, we found that HPV E7 is functionally associated with IRF-1. Binding assays indicate a physical interaction between IRF-1 and HPV E7 in vivo and in vitro. The carboxy-terminal transactivation domain of IRF-1 was required for the interaction. Transient co-expression of E7 significantly inhibits the IRF-1-mediated activation of IFN-β promoter in NIH-3T3 cells. Co-transfection of E7 mutants reveals that the pRb-binding portion of E7 is necessary for the E7-mediated inactivation of IRF-1. It was next determined whether histone deacetylase (HDAC) is involved in the inactivation mechanism as recently suggested, where the carboxy-terminal zinc finger domain of E7 associates with NURD complex containing HDAC. When trichostatin A, an inhibitor of HDAC, was treated, the repressing activity of E7 was released in a dose-dependent manner. Furthermore, the mutation of zinc finger abrogates such activity without effect on the interaction with IRF-1. These results suggest that HPV E7 interferes with the transactivation function of IRF-1 by recruiting HDAC to the promoter. The immune-promoting role of IRF-1 evokes the idea that our novel finding might be important for the elucidation of the E7-mediated immune evading mechanism that is frequently found in cervical cancer.

HPV is strongly implicated as a causative agent in the etiology of cervical dysplasia and cervical cancer (1). Of known HPV subtypes to date, high-risk HPV-16 and HPV-18 are frequently detected in malignant tumors and associated with 70% of all cervical carcinomas (2). In contrast, low-risk HPV-6 and HPV-11 are associated with benign genital warts and are very rarely detected in tumors. The malignant phenotype of high-risk types depends on the expression of two viral oncogenes, E6 and E7. A number of genetic and biochemical studies have shown that E6 and E7 proteins cooperatively exert cellular immortality and transformation by interfering with the functions of the cellular tumor suppressor proteins, p53 and pRb, respectively (3). Of two HPV oncoproteins, E7 is the major transforming protein and is structurally and functionally similar to adenovirus E1A and simian virus T antigen. Based on the similarity, E7 can be divided into three domains: the amino-terminal conserved region 1 (CR1), CR2 containing LXLCXE motif, and the carboxyl-terminal zinc fingers (CR3). Functionally, the CR2 portion of E7 associates with the hypophosphorylated form of pRb and interferes with its binding to E2F. The binding leads to the release of E2F from the pRb-E2F complex, which subsequently activates genes essential for progression through late G1 and S phase, including those necessary for the DNA replication, cell cycle regulation, and proto-oncogenes c-myc and B-myb (4).

In addition to these features of E7, other or additional activities have been reported that are independent of pRb in the course of cellular transformation. For example, the pRb binding-defective mutant of E7 still exerts immortality to primary human genital epithelial cells by cooperating with E6 (5). Moreover, deletion mutants of either CR1 or the carboxy-terminal region of E7 markedly reduce its transforming capacity, although still forming a complex with pRb (6–8). Most recently, it has been shown that the carboxy-terminal zinc finger domain of HPV-16 E7 associates with histone deacetylase (HDAC)1 through the direct interaction with Mi2β, a component of the NURD complex (9). Furthermore, the interaction was implicated in the transcriptional repression of E7 by recruiting HDAC to the promoter of a specific gene, although neither target transcription factor or gene was not identified yet, which is absolutely important for the physiological role of the interaction. These findings collectively suggest that the pRb-independent activities of E7 may be involved in transformation of target cells.

Interferon regulatory factor-1 (IRF-1), originally identified as an interferon (IFN)-β promoter binding transcription factor, has been implicated for a critical mediator of IFN signaling, when induced by various stimuli such as the viral infection, IFNs, retinoic acids, prolactin, and tumor necrosis factor α (TNF-α) (10). Moreover, it has been reported that IRF-1 overexpression inhibits cell growth (11) and that introduction of activated c-Ha-ras oncogene alone is sufficient to transform embryo fibroblasts from IRF-1−/− mice (12). These data suggest that the IRF-1 gene is a tumor suppressor gene that may be associated with the antiproliferative effect of IFN-α.
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Many viral pathogens have evolved various strategies for inhibiting the expression of immune-modulating molecules on infected cells to avoid T lymphocyte attack (13). Adenovirus E1A, homologous to HPV E7, has been demonstrated to inhibit the expression of MCP-1 at the transcriptional level (14). Furthermore, TAP-1 promoter activity is down-regulated in oncogenic adenovirus 12-transformed cell (15). Recently, it also has been reported that HPV-16 E6 binds to the carboxyl-terminal transactivation domain of IRF-3, another member of the IRF family, and inactivates its transactivation function (16). Therefore, it could be speculated that HPV E6 or E7 oncoprotein might interfere with IFN signaling, and thus lead to evading host immunity in the progress of cervical carcinogenesis.

To see whether IRF-1 is a cellular target of HPV E7 oncoprotein involved in the perturbation of host IFN signaling, we performed a cellular interaction assay. GST pull-down assay confirmed that IRF-1 could directly interact with HPV-16 E7, which was initially demonstrated by yeast and mammalian two-hybrid assay (17). We further show that HPV E7 abrogates the transactivation function of IRF-1 by direct interaction, not by its targeting for degradation. Studies with HDAC inhibitor and E7 mutants suggest that the transrepression activity of E7 be mediated by recruiting HDAC to the IFN-β promoter through the interaction with IRF-1, and that the pRb-binding portion (CR2) of E7 is necessary, but needs additional zinc finger region (CR3) for the sufficient suppression. Our novel finding could improve the understanding of the molecular basis of immune evasion strategy used by the HPV E7 in cervical carcinogenesis.

EXPERIMENTAL PROCEDURES

Plasmids—Details on individual plasmid constructs, which were verified by sequencing, are available on request. Yeast expression plasmids for two-hybrid assays, pBTM116 (Tpt1: LexA DNA binding domain vector) (17) and pASV3 (Leu2: VP16 acidic transactivation domain vector) (18), are multicopy and are expressed under the control of ADH1 and PGR promoters, respectively. IRF-1 expression plasmid was kindly provided by Dr. Tucker Collins (Boston, MA) and modified by re-cloning into yeast vectors for two-hybrid assay and pSG5 vector (Stratagene, La Jolla, CA) for transient cotransfection assay. IFN-β-CAT reporter plasmid was kindly provided by Dr. J. Hiscott (Quebec, Canada). Expression plasmids for GST-E7 and E7 mutants were kindly provided by Drs. S. H. Lee (Chuncheon, Korea) and D. A. Galloway (Seattle, WA), respectively. Ga14-E7 fusion protein was expressed from a pG4M-polyII vector (19).

Yeast Two-hybrid Assay—This assay was done as described previously (20). Briefly, cDNA sequence of IRF-1 was introduced into the vector pBTM116, while cDNA sequences of HPV-16 E7, −11 E7, −16 E6, and −16 E6/E7 were introduced into the vector pASV3. The yeast Saccharomyces cerevisiae L40 strain (MATa, his3A2000, trpl1–901, leu2–3, 112, ade2, lys2::(lexAop)2-HIS3, ura3::(lexAop)2-lacZ) (a gift from S. M. Hollenberg, Dept. of Cell and Developmental Biology, Oregon Health Sciences University) (17) was transformed with a pBTH116 plasmid and a pASV3 plasmid one by one by the lithium-acetate procedure (21). Transformants were grown in 15 ml of minimal liquid medium supplemented with histidine (50 mg/liter) and adenine (20 mg/liter). Extracts were prepared by a freezing-thawing lysis technique (22) and analyzed for β-galactosidase activity according to a standard procedure (21), except that reactions were performed in 96-well plates.

Mammalian Two-hybrid Assay—To confirm the interaction in the mammalian cell, transient transfections were performed in NIH-3T3 cells using (17 mer), TATA-CAT reporter plasmid, Gal4 (DBD)-E7, and VP16 (AD)-IRF-1 expression vectors. Fold stimulation was calculated from the amount of CAT enzyme determined by CAT ELISA.

FIG. 1. The interaction of IRF-1 with HPV E7. A, HPV-16 E7 specifically interacts with IRF-1 in yeast. B, both HPV-16 and HPV-11 E7 interacts with transactivation activation domain (TAD) of IRF-1. Combinations of LexA DNA-binding domain fusions (IRF-1, IRF-1[(1–166)], IRF-1[(217–325)]) and VP16 transactivation domain fusions (16E6, 16E7, 11E7, 16E6/E7) were shown. The strength of interaction was analyzed by β-galactosidase assay. Units (U) are expressed in nmol/min/mg of total protein.

C, physical interaction of HPV-16 E7 with IRF-1 in vitro. The GST pull-down assay was performed using purified GST-E7 protein and in vitro translated 35S-labeled IRF-1. L indicates loading material used for binding assay. D, the interaction of IRF-1 with HPV E7 in mammalian cells. Transient transfections were performed in NIH-3T3 cells using (17 mer), TATACAT reporter plasmid, Gal4 (DBD)-E7, and/or VP16 (TAD)-IRF-1 expression vector. After transfections, cells were harvested and extracted. The protein concentration and β-galactosidase activity were determined for the normalization of the CAT assay. Fold stimulation was calculated from the amount of CAT enzyme determined by CAT ELISA.

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by dividing the amount of CAT enzyme, determined by CAT ELISA, of Gal4- and VP16-treated cells by that of Gal4-control cells.

**GST Pull-down Assay**—In vitro binding studies using GST-fused E7 were performed as described with a little modification (23). Briefly, GST or GST-E7 proteins were expressed in *Escherichia coli* and purified on glutathione-Sepharose (Amersham Pharmacia Biotech). Wild type IRF-1 was in vitro translated in 50 µl of rabbit reticulocyte lysate (Promega) supplemented with [35S]methionine (Amersham Pharmacia Biotech). Glutathione-Sepharose beads were equilibrated with binding buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 µM ZnCl2, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet-40). An approximately equimolar amount of GST (0.5 µg) or GST-E7 (1 µg) was mixed with 15 µl of [35S]-IRF-1, and the mixtures were incubated at room temperature for 30 min. Pre-equilibrated glutathione-Sepharose beads were added and further incubation was allowed for 1 h. The beads were washed three times with the binding buffer. Bound proteins were detected by SDS-polyacrylamide gel electrophoresis and autoradiography.

**In Vitro Degradation Assays**—Either 15 µl of [35S]methionine-labeled IRF-1 synthesized in vitro in rabbit reticulocyte lysate was mixed with GST (0.5 µg) or GST-E7 (1 µg) in the presence of 1× phosphate-buffered saline buffer containing 2 mM dithiothreitol. The reaction mixtures were incubated at room temperature. At various times, aliquots were removed, mixed with an equal volume of 2× SDS-loading buffer, and analyzed by autoradiography.

**Transient Transfection and CAT ELISA**—Transfection of cells was carried out by CaPO4/DNA precipitation as described (24). NIH-3T3 cells (~104), maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, were plated in 60-mm dishes 5 h before transfection. Total amounts (6 µg) of transfected DNA including SV40-driven β-galactosidase internal control plasmid were kept constant by including the respective control plasmids without insertions as indicated. After overnight transfection, cells were washed, fed with the complete medium, and further incubated for 24 h. Cells were washed twice with ice-cold phosphate-buffered saline, collected, resuspended in 100 µl of 0.25 M Tris-HCl (pH 7.6), and subjected to three free-thaw cycles. Samples were cleared by centrifugation (12,000 rpm, 10 min, 4°C), and protein concentrations were determined with the Bio-Rad protein assay dye reagent. 30–70 µl of the clear lysates was tested for CAT concentration in the CAT ELISA according to the instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The CAT concentration of each sample was normalized with respect to β-galactosidase activity. All the data presented in the text represent the mean of at least three independent transfections. Fold stimulation was calculated by dividing the amount of CAT enzyme of treated cells by that of untreated cells.

**FIG. 2.** HPV-E7 interferes with transcriptional activity of IRF-1. Mouse NIH-3T3 cells were transfected with combinations of IRF-1, HPV-16, and/or HPV-11 E7 expression vectors together with IRF-1-responsive IFN-β promoter-CAT reporter plasmid. Data represent the mean ± SD of at least three independent transfections. Fold stimulation was calculated by dividing the amount of CAT enzyme of treated cells by that of untreated control cells.

**FIG. 3.** The amino-terminal domain of HPV-16 E7 is critical for the repression of IRF-1. A, the structure of HPV-16 E7 protein, where two amino-terminal deletion mutants of E7 are shown. B, transient transfections were performed in NIH-3T3 cells using IFN-β promoter-CAT reporter and indicated E7 mutants (E7wt, wild type; E7Δ 6–10, deletion of amino acids 6–10; E7Δ 21–24, deletion of amino acids 21–24, defective in pRb-binding). CAT ELISA was performed as described. C, the amino-terminal domain of HPV-16 E7 is required for the interaction with IRF-1. Yeast two-hybrid and β-galactosidase assays were performed as described.
RESULTS
HPV E7 Protein Interacts with IRF-1—The interaction in vivo between HPV E7 and IRF-1 was analyzed by the yeast two-hybrid assays for which full-length IRF-1 was fused to the LexA DNA-binding domain, and HPV E7 was fused to the VP16 acidic activation domain. Because the yeast strain L40 contains a LexA DNA-binding domain, and HPV E7 was fused to the VP16 transcriptional activity when compared with LexA without fusion. When both LexA-fused IRF-1 and VP16-fused HPV-16 E7 were introduced, an ~5-fold increase of β-galactosidase activity was observed, indicating that E7 interacts with IRF-1 in yeast cells. However, no interaction was detected when VP16-E6 was introduced, and the expression of E6 did not influence the interaction between E7 and IRF-1. Reversed experiments to confirm above results could not be carried out because the expression of VP16-fused IRF-1 was toxic to the yeast L40. Deletion of the carboxyl-terminal transactivation domain (amino acid residues 167–325) of IRF-1 abolished the interaction (Fig. 1B). Interestingly, the carboxyl-terminal domain of IRF-1 was found to interact with both high-risk HPV-16 and low-risk HPV-11, suggesting that E7 interacts with IRF-1 without type-specificity of HPV.

To determine whether the interaction is direct, we performed an in vitro binding assay for which GST or GST-E7 protein was induced in E. coli and purified and then mixed with in vitro translated [35S]methionine-labeled IRF-1 in rabbit reticulocyte lysate. Binding of E7 to IRF-1 was assessed by GST pull-down assay. As shown in Fig. 1C, about 10% of input IRF-1 protein was retained on the E7-conjugated Sepharose beads, confirming that IRF-1 binding to HPV E7 is direct in vitro.

Because E7 antibody is not so available for immunoprecipitation that is frequently used to demonstrate that the interaction is physiological in mammalian cells, we instead carried out a mammalian two-hybrid assay. When Gal4-E7 or VP16-IRF-1 alone was expressed in NIH-3T3 cells, the expression of CAT reporter gene was marginal. However, the CAT expression was increased to 3.5-fold by coexpression of Gal4-E7 and VP16-IRF-1 (Fig. 1D), suggesting that the interaction is physiological in vivo.

HPV E7 Abrogates the Transactivation Function of IRF-1—Given the physical interaction between E7 and IRF-1 as described, we next measured the effect of E7 expression on the transactivation activity of IRF-1 in NIH-3T3 cells using an IFN-β promoter-CAT reporter gene construct. Expression of IRF-1 resulted in up-regulated transcription from this promoter, and co-expression of HPV-16 E7 inhibited the IRF-1-directed activity in a dose-dependent manner (Fig. 2). As expected from the interaction assays, low-risk HPV-11 E7 also abrogated the transactivation function of IRF-1, indicating that HPV-11 also needs to inactivate host immunity presented by IRF-1 for the formation of a benign tumor such as a genital wart.

pRb-binding Motif of E7 Is Essential for the Transrepression of IRF-1 Function—To determine that the pRb-binding LXCXE motif of E7 is responsible for the inactivation of IRF-1, two deletion mutants of E7 were used for transfection and binding assays. As depicted in Fig. 3A, Δ6–10 is a transformation-defective mutant without affect to pRb binding and Δ21–24 (LXCXE motif) is completely defective for pRb binding as well as transformation (25). Coexpression of wild type E7 abolished the transactivation function of IRF-1 in a dose-dependent manner. However, Δ6–10 and Δ21–24 mutants showed little repression and no repression against IRF-1 activity, respectively (Fig. 3B). This result suggests that either pRb mediates the repressing activity of E7 or, more directly, the region of E7 is required for interaction with IRF-1.

Yeast two-hybrid assay was performed to demonstrate that the pRb-binding portion of E7 is critical for the interaction with IRF-1. As shown in Fig. 3C, the repression-defective Δ21–24 mutant was not capable of interacting with IRF-1. E7 Δ6–10, a weakly repressing mutant, showed little binding activity to IRF-1. These results suggest that the repression activity of E7 is correlated to the binding of E7 to IRF-1. Therefore, it is likely that the CR1/2 containing pRb-binding portion of E7 is functionally important for mediating E7-directed transrepression of IRF-1 function through the direct protein-protein interaction.

How HPV E7 Inactivates IRF-1—To determine how HPV E7 inactivates the transactivation function of IRF-1, we performed three independent assays. From in vitro degradation assay and gel mobility shift assay, we could not observe any effects of E7 on the degradation and DNA binding activity of IRF-1, respectively (data not shown). Finally, when we treated TSA, an inhibitor of HDAC, to the cells transfected with E7 and IRF-1 expression vectors and IFN-β promoter CAT reporter, we found that the E7-mediated repression of IRF-1 activity was released in a dose-dependent manner of TSA (Fig. 4). Based on the report that HPV E7 is functionally associated with transcriptional repression NuRD complex containing HDAC, our observations suggest that IRF-1 could be one of repression target of HPV E7. To further confirm these results, we analyzed E7 mutants that are mutated in the carboxyl-terminal zinc finger domain. It has been reported that C58G/C91G mutant is defective in recruiting HDAC, whereas S71I mutant is not (Fig. 5A) (9). As expected, both mutants are able to interact with IRF-1, probably through the amino-terminal pRb-binding domain (Fig. 5B). However, the transcription assays indicate that C58G/C91G mutant loses its repressing activity, but S71I is still capable of repressing IRF-1 function (Fig. 5C). These results indicate that the carboxyl-terminal zinc finger domain of E7 is required for the E7-mediated repression of IRF-1, probably by recruiting HDAC to IRF-1 response region.
DISCUSSION

A number of biochemical and genetic studies have suggested that HPV-16 E7 plays a critical role in the development of cervical cancer by inactivating pRb. However, recent evidence has indicated that inactivation of pRb is necessary but not sufficient for transformation (5–8), suggesting that in addition to pRb, another cellular target of E7 is required for its full transforming activity. As depicted in Fig. 6, our results could indicate pRb-independent function of E7 in two aspects: (i) E7 interacts with the carboxyl-terminal transactivation domain of IRF-1 and inactivates its transactivation function; (ii) inactivation requires both the amino-terminal domain of E7, including pRb-binding pocket, for binding to IRF-1 and the carboxyl-terminal zinc finger for recruiting HDAC independent of pRb. Recently, it has been shown that HPV-16 E7 functionally associates with HDAC and thus represses transcription. However, any target transcription factors or genes should be identified for the interaction to be physiologically relevant. Our data indicate that IRF-1 is a novel cellular target of E7 for transcriptional repression for which both domains of E7 are required. Furthermore, a mechanism underlying the inactivation is likely because of recruitment of HDAC by CR3 zinc finger domain of E7 to IRF-1. These overall suggestions might be physiologically related to the fact that both domains are essential for the complete transforming activity of HPV E7 in vivo.

Immunity evasion is a process to be required for the progress of all malignant tumors. Both MHC molecules and TAP-1 are essential for the T lymphocyte recognition of antigen through the formation of MHC-peptide complexes (26). MCP-1, a chemottractant molecule, is responsible for the attraction of T lymphocyte to the target cells (27). Molecular epidemiological studies have suggested that HPV-caused development of cervical cancer might be closely related to the escape of host immune surveillance. In HPV-infected cervical cancer tissue, the expression of MHC class molecules and TAP-1 are markedly down-regulated (28, 29). In addition, MCP-1 is much less expressed than in the stroma surrounding the carcinoma cells (30, 31). These immune-modulator genes are transcriptionally induced either directly by IFN-activated STAT or indirectly by IFN-induced IRF-1 (32–36). Thus, the inactivation of either STAT or IRF-1 could be one mechanism by which HPV escapes host immune surveillance. In agreement, some molecular evidence has been reported as followed. HPV-16 E6 binds to the carboxyl-terminal transactivation domain of IRF-3 and inhibits its transcriptional activity without targeting for proteasome-mediated degrading (16). IRF-3 is part of a virus-activated transcription factor, of which activity is increased in response to viral infection (37), and is involved in type I IFN gene expression (38). Recently, HPV-16 E7 has been implicated in immune evasion by inducing E7-specific cytotoxic T cell tolerance (39) and by abrogating IFN-α-mediated signaling (40) although the molecular mechanism has not been clearly determined. In this study, we showed that HPV E7 physically interacts with IRF-1, which is critical for IFN signaling, and abrogates its transactivation function, probably through histone deacetylation. Therefore, our novel observation could contribute to elucidation of the molecular mechanism of E7-mediated immune evasion. However, the in vivo implication of the inactivation remains to be determined. Detailed analysis of these works is currently under way in our laboratory, for example construction of tetracycline-inducible E7 expressing cell line.

The functional inactivation of IRF-1 by both high-risk (HPV-16 and HPV-18) and low-risk HPV (HPV-6 and HPV-11)
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E7s could play an important role in the malignant transformation and benign condyloma formation of cervix, respectively. Once high-risk HPV infects, it produces E6 and E7 oncoproteins. Both inactivate two cellular tumor suppressor proteins by which E6 binds to p53 and promotes its proteolysis and E7 binds to hypophosphorylated form of pRb and interferes with its binding to E2F. Thus, the inactivation of IRF-1 tumor suppressor by high-risk E7 would provide another advantage to a cervical carcinogenesis. However, unlike pRb, IRF-1 could be inactivated by low-risk HPV-11 E7 as demonstrated in our assay. Although the physiological implication of such an observation is not clear at present, it is tempting to speculate that IRF-1 could be one of target growth regulators of low-risk E7 involved in the benign tumor formation such as a cervical condyloma. In this regard, the development of a peptide drug to break the E7-IRF-1 interaction could be used as a therapeutic agent for the treatment of malignant and benign cervical lesions.