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Human Papillomavirus Type 16 E5 Protein Induces Expression of Beta Interferon through Interferon Regulatory Factor 1 in Human Keratinocytes▽

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Crucial steps in high-risk human papillomavirus (HR-HPV)-related carcinogenesis are the integration of HR-HPV into the host genome and loss of viral episomes. The mechanisms that promote cervical neoplastic progression are, however, not clearly understood. During HR-HPV infection, the HPV E5 protein is expressed in precancerous stages but not after viral integration. Given that it has been reported that loss of HPV16 episomes and cervical tumor progression are associated with increased expression of antiviral genes that are inducible by type I interferon (IFN), we asked whether E5, expressed in early phases of cervical carcinogenesis, affects IFN-β signaling. We show that the HPV type 16 (HPV16) E5 protein expression *per se* stimulates IFN-β expression. This stimulation is specifically mediated by the induction of interferon regulatory factor 1 (IRF-1) which, in turn, induces transcriptional activation of IRF-1-targeted interferon-stimulated genes (ISGs) as double-stranded RNA-dependent protein kinase R (PKR) and caspase 8. Our data show a new and unexpected role for HR-HPV E5 protein and indicate that HPV16 E5 may contribute to the mechanisms responsible for cervical carcinogenesis in part via stimulation of IFN-β and an IFN signature, with IRF-1 playing a pivotal role. HPV16 E5 and IRF-1 may thus serve as potential therapeutic targets in HPV-associated premalignant lesions.

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that infect cutaneous and mucosal epithelial tissues in several ano-genital and skin regions and the tracheo-bronchial and oral mucosa. Ninety-nine percent of cervical cancers are positive for HPV DNA, and a subset of HPVs, known as high-risk (HR) types, including HPV16 and HPV18, is primarily associated with cancer development (57).

A critical step in cervical neoplastic progression is the integration of HPV DNA into the host genome (33, 57). Integration is associated with deletion of regions, including the El, E2, E4, and E5 open reading frames (ORFs), while E6 and E7 genes, together with the upstream regulatory region, are retained, and their deregulation, related to high-level expression throughout the epithelium, represents the main determinant of progression toward the malignant phenotype (47).

The mechanisms that promote cervical neoplastic progression are not clearly understood. Recently, it has been reported that spontaneous loss of episomes in W12 cells, a unique model of progression of HPV16-related cervical neoplasia, is associated with increased expression of antiviral genes that are inducible by type I interferon (IFN) (37). Accordingly, the treatment of W12 cells with IFN-β can dramatically accelerate the progression from an ostensibly episomal population to one in which only integrants remain (21).

IFN-β is produced upon infection in many cell types and induces an antiviral state through paracrine IFN production and the subsequent activation of interferon-stimulated genes (ISGs) (49). During viral infection, transcriptional induction of the *IFN*-β gene is mediated by induction and/or activation of transcription factors of the IFN regulatory factor (IRF) family, specifically, IRF-1, IRF-3, and IRF-7, which bind to a motif termed the interferon regulatory factor element (IRF-E), also known as the IFN-stimulated response element (ISRE), present in the promoter of ISGs (22, 54).

IRF-1 was originally identified as a regulator of the virus-inducible enhancer-like element of the human *IFN*-β gene (20), but it was then recognized as being able to regulate several ISGs and amplify the IFN response (28). IRF-1 is expressed at low levels in most cell types and is upregulated by different stimuli. Conversely, IRF-3 is constitutively expressed in the cytosol and, upon infection, is suddenly activated by phosphorylation that leads to its nuclear translocation and subsequent induction of target genes. IRF-7 is expressed at a low level in most cell types, where its expression can be induced by type I IFN. Similarly to IRF-3, IRF-7 is activated through phosphorylation, undergoes nuclear translocation, and is involved in the second wave of sustained IFN-α/β production (22).

IRF-1 and IRF-3 are well-known targets of HPV16 E6- and E7-mediated inhibition of host defense responses (4, 36, 42). Less investigated is the role, in IFN system modulation, of the HPV16 E5 oncoprotein.

HPV16 E5 is a hydrophobic protein of 83 amino acids that associates with the Golgi apparatus, the endoplasmic reticulum, and nuclear membrane (17). E5 exhibits transforming activity (52) and cooperates with E6 and E7 to induce a malignant phe-

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notype (6, 51, 56). E5 is also able to modulate several cellular pathways through activation of the epidermal growth factor receptor (EGFR) in a ligand-dependent manner (11, 55). It has been demonstrated that through EGFR, E5 can upregulate vascular endothelial growth factor (26) and can enhance cyclooxygenase 2 expression (27). E5 also has a primary role in the productive phase of the viral life cycle (12, 18) and in the proliferative capacity of HPV-positive cells upon differentiation (40). Before integration, when the HPV genome is episomal, the E5 mRNA is the most abundant viral transcript (50); however, it is no longer expressed by the integrated HPV DNA (47), suggesting that unlike E6 and E7, which are clearly associated with the transformed phenotype, E5 might play an important role in the early phases of tumorigenesis. Since spontaneous loss of episomes and selection of integrants have been associated with an IFN signature (21, 37), we sought to investigate whether, among HPV early proteins expressed before viral integration, E5 was able to impact IFN signaling.

In this paper, we provide evidence that E5 functions as a positive regulator of *IFN*-β gene expression through the induction of IRF-1. We also demonstrate that E5-induced IRF-1 expression results in transcriptional activation of IRF-1-targeted ISGs.

These findings indicate that the HPV16 E5 protein, through stimulation of the type I IFN response, may contribute to the loss of viral episomes in infected cervical keratinocytes, accelerating the progression to invasive cancer. Moreover, our data identify HPV16 E5 and IRF-1 as potential therapeutic targets in HPV-associated premalignant lesions in order to limit selection of integrants.

MATERIALS AND METHODS

Cell lines and treatment. The spontaneously immortalized human keratinocyte cell line HaCaT has been stably transfected with the empty vector pMSG (HaCaT/pMSG) or HPV16 *E5* gene (HaCaT/E5), under the control of the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter (35), and these cells were a generous gift of A. Alonso (German Cancer Research Centre, University of Heidelberg, Heidelberg, Germany). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and penicillin-streptomycin (Gibco, Karlsruhe, Germany) to 70 to 80% confluence. Cells were serum starved for 24 h and induced with 1 μM dexamethasone for 24 h or different times, as indicated below. C33A cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 containing 10% fetal bovine serum, glutamine, and penicillin-streptomycin. The W12 cell line is derived from a cervical intraepithelial neoplasia (48). The 20863 (W12E) and 20861 (W12G) subclones have been previously described (23) and were a generous gift of A. Venuti (Istituto Regina Elena, Rome, Italy).

Western blot analysis. Whole-cell extracts (50 μg) were prepared as previously described (10). Cytoplasmic and nuclear extracts (50 μg) were obtained by using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Inc., Waltham, MA). Proteins were separated by 7.5% or 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, United Kingdom). Blots were incubated with anti-IRF-1 (1:200; sc-497; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-IRF-3 (1:200; sc-9082; Santa Cruz Biotechnology), anti-phospho-IRF-3 (1:1,000; 4947; Cell Signaling Technology, Inc., Beverly, MA), anti-IRF-7 (1:200; sc-15994; Santa Cruz Biotechnology), anti-protein kinase R (PKR; 1:200; sc-707; Santa Cruz Biotechnology), anti-caspase 8 (1:200; 9746; Cell Signaling), anti-USF-2 (1:200; sc-862; Santa Cruz Biotechnolgoy), anti-β-actin (1:200; sc-47778; Santa Cruz Biotechnology), and anti-rabbit- or anti-mouse–horseradish peroxidase–coupled secondary antibody (Amersham, Buckinghamshire, United Kingdom). Immune complexes were identified by using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom). The intensities of specific bands were measured by densitometry and are reported as the increase in relative expression in treated cells compared with control cells.

Real-time RT-PCR. Total RNA was isolated by the RNAzol B method (Biotecx Laboratories, Houston, TX), and it was reverse transcribed (2 μg) using Moloney murine leukemia virus reverse transcriptase and oligo(dT) priming according to manufacturer's protocol (Retroscript Ambion, Austin, TX). Real-time reverse transcription-PCR (RT-PCR) was run in a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN) using the LightCycler Fast-Start DNA Master SYBR green I kit (Roche Diagnostics, Indianapolis, IN) with the following protocol: initial activation of HotStar *Taq* DNA polymerase at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, primer-specific annealing for 15 to 30 s at different temperatures, and elongation at 72°C for 10 s. The following primers were designed for cDNA amplification: for E5, For (5′-CCACAACATTACTGGCGTGC-3′) and Rev (5′-GCAGAGGCTGCTGTTATCCAC-3′); for IFN-β, For (5′-GCAGCAGTTCCAGAAGGAG-3′) and Rev (5′-GCCAGGAGGTTCTCAACAAT-3′); for IRF-1, For (5′-AGCTCAGCTGTGCGAGTGTA-3′) and Rev (5′-CATGACTTCCTCTTGGCCTT-3′); for caspase 8, For (5′-AGAGCCTAGGAGAGCGATG-3′) and Rev (5′-CACCATCAATCAGAAGGGAAG-3′); for PKR, For (5′-CGATACATGAGCCCAGAAAC-3′) and Rev (5′-GTTTCAAAAGCAGTGTCAC-3′). Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal control, were For (5′-CCAAAATCAAGTGGGGCGATG-3′) and Rev (5′-AAAGGTGGAGGAGTGGGTGTCG-3′). For each cDNA, we determined housekeeping gene and target gene sequences in triplicate. The comparative threshold cycle (C_T) method was used to determine the increase of transcript present in E5-expressing cells compared to controls (38).

Transfection experiments and enzymatic assays. Transfection experiments were performed using the Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Cells were transfected with the following constructs: the construct encoding the *IFN*-β gene promoter/enhancer cloned upstream of the luciferase reporter gene (29), the construct for p3500 (encoding the entire IRF-1 promoter from bp −34000 to +168), the three portions of Gas/κB (corresponding to the −199 to −89 region of the IRF-1 promoter), GAS (the −126 to −113 region of the IRF-1 promoter), and NK-κB (the −89 to −16 region of the IRF-1 promoter), all cloned upstream of the luciferase reporter gene (39); the construct pISRE-TA-luc (Invitrogen, Carlsbad, CA), containing five copies of consensus IRF-E sequence upstream of the firefly luciferase gene; IRF-1-expressing vector (29); E5 expression vector (3). Cells were transfected with 500 ng of luciferase reporter constructs and 500 ng of expression vectors. The amount of transfected DNA was adjusted with the empty vector RcCMV. One hundred nanograms of pAct-*Renilla* plasmid was cotransfected and used as a control for transfection efficiency. Reagents from Promega were used to assay extracts for luciferase activity in a Lumat LB9501 luminometer (E&G Berthold, Bad Wildbad, Germany).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (13, 41). Briefly, HaCaT/E5 and HaCaT/pMSG cells treated for 24 h with dexamethasone and W12E/G cells were cross-linked with 1% formaldehyde and quenched in 0.125 M glycine. The cell lysates were sonicated and immunoprecipitated with normal rabbit serum (BD) or anti-IRF-1-specific antibodies (Abs; Santa Cruz Biotechnology). The immunoprecipitated DNA was eluted and amplified by real-time PCR by using an ABI 7700 system (Applied Biosystems). Values were normalized to the corresponding input control and are expressed as the relative level of binding to normal rabbit serum for each experiment. The sequences of specific primers used for amplification of the human *IFN*-β (*hIFN*-β) gene were as follows: For, 5′-GTCATTCACTGAAACTTTA-3′; Rev, 5′-AGGTTGCAGTTAGAATGTC-3′. Real-time PCR was performed as previously described (13).

Knockdown of IRF-1 expression. IRF-1 small interfering RNA (siRNA) oligonucleotide target sequences were selected and inserted in retroviral vectors as described previously (46). The ability of the DNA construct to silence IRF-1 expression was assessed by Western blotting using 50 μg of whole-cell extracts from HaCaT/E5 cells harvested 48 h after transfection with IRF-1 siRNA or luciferase siRNA and treated for 24 h with dexamethasone.

Neutralizing assay. HaCaT/pMSG and HaCaT/E5 cells were treated or untreated with 1 μM dexamethasone and 500 IU/ml of IFN-β1a (Rebif; Serono, Geneva, Switzerland) for 24 h in the presence or absence of 1,000 IU/ml sheep antiserum raised against human IFN-α and IFN-β (PBL Biomedical Laboratories, New Brunswick, NJ). The E5-mediated expression of IRF-1 was assessed by Western blotting as described above.

Enzyme-linked immunosorbent assay. To measure cell-associated IFN-β, cells were lysed in buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 30 mM $Na_4P_2O_7$, 10% glycerol, 1 mM benzamidine, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. One hundred micrograms of total protein extract was then analyzed to determine the level of IFN-β by using a sensitive enzyme-linked immunosorbent

assay (ELISA; PBL InterferonSource, Piscataway, NJ) according to the manufacturer's instructions. The results are expressed in pg/100 μg of total proteins.

Statistical analysis. All the experiments were repeated at least three times. Student's *t* test was used to evaluate statistical differences between means. *P* values of ≤0.05 were considered statistically significant.

RESULTS

The HPV16 E5 protein induces IFN-β expression. HPV16 E5 expression in dexamethasone-treated HaCaT/E5 cells was assessed by real-time RT-PCR of E5 transcripts, since no antibodies to the protein are available. E5 transcripts were not detected in dexamethasone-treated HaCaT/pMSG control cells, whereas enhanced levels of E5 transcripts were present in dexamethasone-treated HaCaT/E5 cells. E5 expression also appeared in dexamethasone-untreated HaCaT/E5 cells, indicating that, as reported elsewhere (35), in the absence of the inducer the MMTV promoter was not completely shut off (Fig. 1A).

We investigated IFN-β expression in HaCaT/E5 cells compared with HaCaT/pMSG control cells. As shown in Fig. 1B, induction of E5 produced a 12-fold increase in IFN-β mRNA accumulation upon dexamethasone treatment (lane 4 versus lane 2). This finding mirrored a substantial enhancement in IFN-β promoter activity (13-fold increase) in E5-expressing cells compared with control cells (Fig. 1C, lane 4 versus lane 2). Increased IFN-β mRNA accumulation and promoter activity also appeared in dexamethasone-untreated HaCaT/E5 cells (Fig. 1B and C, lane 3), in accordance with detectable E5 expression in the absence of the inducer (Fig. 1A, lane 2).

We next correlated the kinetics of E5 expression with *IFN*-β gene induction. RNA was isolated from HaCaT/E5 cells treated with dexamethasone for 6, 12, or 24 h and then analyzed by real-time RT-PCR. Along with enhanced expression of E5 mRNA for up to 24 h (Fig. 1D; 3.5-fold increase), IFN-β mRNA basal expression was increased during the treatment time (Fig. 1E; 6.5-fold increase).

To confirm these findings in another system not dependent on dexamethasone, we transiently transfected an E5 expression vector in C33A human keratinocytes and assessed endogenous IFN-β expression and *IFN*-β gene promoter activity in comparison to cells transfected with the empty vector. As shown in Fig. 2A, dose-response experiments indicated a specific E5-mediated increase in IFN-β mRNA accumulation of up to 6-fold. Similarly, a substantial enhancement in IFN-β promoter activity was observed with increasing doses of E5-expressing vector compared with cells transfected with the empty vector (Fig. 2B).

To study *IFN*-β gene induction by the HPV16 E5 protein in a more natural setting, we performed experiments in the HPV16-positive cervical epithelial cell line W12, a unique model of cervical squamous carcinogenesis. In particular, two selected clones of W12 were used: W12E, in which several copies of the genome are maintained episomally (clone 20863, a *bona fide* model for the productive infection), and W12G, which contains only integrated genomes (clone 20861, a *bona fide* model for virus-transformed cells) (23). E5 expression in these cells was assessed by real-time RT-PCR and, as shown in Fig. 2C, only W12E cells expressed E5 at high levels (~30-fold) compared with dexamethasone-induced HaCaT/E5 cells. Conversely, no detectable levels of E5 were present in W12G cells. Consistently, a substantial stimulation of IFN-β mRNA accumulation was observed only in W12E cells, while it was repressed in W12G cells (Fig. 2D).

We also measured IFN-β protein production in HaCaT and W12 cells. Since secreted IFN-β protein was not detected by ELISA in conditioned medium of HaCaT or W12 cells, as already observed with transforming growth factor β (34), we measured intracellular production of IFN-β in cell lysates by ELISA (Fig. 2E). Consistent with the results obtained at the mRNA level, production of IFN-β was substantially and reproducibly increased in both dexamethasone-treated HaCaT/E5 and W12E cells.

Together, these results indicate that, in human keratinocytes, HPV16 E5 expression correlates with an increased IFN-β expression mediated by enhanced transcriptional activity.

The HPV16 E5 protein does not modulate IFN-β master regulator activity. To shed light on mechanisms leading to E5-mediated IFN-β induction, we investigated expression and activity of IFN master regulators, i.e., IRF-3 and IRF-7, in HaCaT cells treated with dexamethasone for 24 h. Western blot analysis indicated that E5 did not affect basal IRF-3 expression (Fig. 3A). We then examined activation of IRF-3 by blotting with specific antibodies that recognize the phosphorylated protein form. As shown in Fig. 3B, no phosphorylated IRF-3 was detected in E5-expressing cells, which was instead observed in 293 cells transfected with IKK-ε, the IRF-3-activating kinase. A dexamethasone inhibitory effect on IRF-3 activation, which was recently reported (32), was excluded since the discrete amounts of E5 produced in the absence of the drug, while able to induce IFN-β expression (Fig. 1B and C, lane 3), did not activate IRF-3 (Fig. 3B, lane 4). Similar to what was observed for IRF-3, IRF-7 activation, as assessed by detection of nuclear translocation of the protein, was not detected in E5-expressing cells (Fig. 3C).

The HPV16 E5 protein induces IRF-1 expression. IRF-1 mRNA and protein expression levels in dexamethasone-treated HaCaT/E5 and W12E/G cells were evaluated by real-time RT-PCR and Western blot analysis, respectively. These analyses showed that IRF-1 levels were significantly increased in HaCaT/E5 cells compared with the HaCaT/pMSG control cells (Fig. 4A [2-fold increase] and B [4.2-fold increase]). Substantial induction of IRF-1 by E5 was also confirmed in W12 clones, in which high levels of IRF-1 expression were present only in E5-expressing W12E cells, compared with W12G cells (Fig. 4C and D).

To determine whether the HPV16 E5 protein was able to induce IRF-1 promoter activity, HaCaT/E5 cells were transiently transfected with a 3,500-bp IRF-1 promoter construct cloned upstream of the luciferase reporter gene. Basal activity of the IRF-1 promoter showed a 2.5-fold increase (upper value) in E5-expressing cells compared to empty vector-expressing cells (Fig. 4E, lane 2 versus lane 1). To assess which sequences on the IRF-1 promoter were involved in the observed effect, both cells were transfected with fragments of the IRF-1 promoter containing either consensus binding sites for STAT1 (GAS) or NF-κB transcription factors, or a portion containing overlapping binding sites for STAT1 and NF-κB (GAS/κB). As shown in Fig. 4E, basal activation of constructs bearing GAS/κB (lane 4 versus lane 3 [5-fold increase]), GAS (lane 6 versus lane 5 [2-fold increase]), or NF-κB (lane 8 versus lane 7 [3-fold increase]) elements was significantly increased in

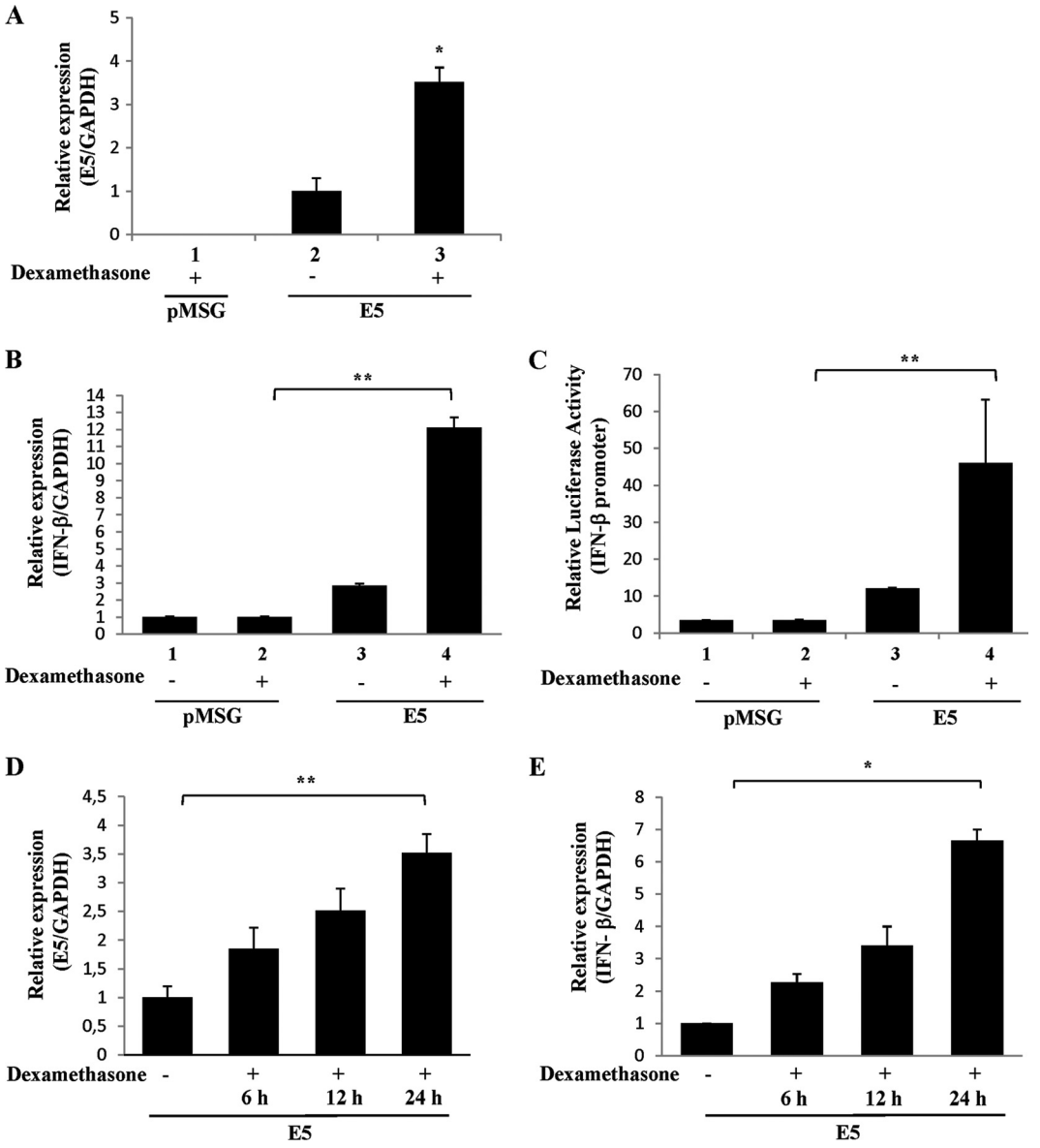

FIG. 1. HPV16 E5 induces IFN-β expression in HaCaT cells. (A) Real-time RT-PCR analysis was performed for the *E5* gene in HaCaT/pMSG (lane 1) and HaCaT/E5 cells without (-) or with (+) dexamethasone treatment. Data were normalized by the levels of GAPDH expression in each sample and are shown as relative expression units. Levels from dexamethasone-untreated HaCaT/E5 cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. *, $P < 0.05$. (B) Real-time RT-PCR analysis was performed for the *IFN*-β gene in HaCaT/pMSG (lanes 1 and 2) and HaCaT/E5 cells (lanes 3 and 4) with (+) or without (-) dexamethasone treatment. Data were normalized as for panel A. Levels from untreated HaCaT/pMSG cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. **, $P < 0.01$. (C) Dexamethasone-untreated or treated HaCaT/pMSG (lanes 1 and 2) and HaCaT/E5 cells (lanes 3 and 4) were transfected with an IFN-β–luc reporter. Luciferase activity was measured 24 h after transfection. Means ± standard deviations from three separate experiments were calculated after normalization with the pAct *Renilla* activity. **, $P < 0.01$. (D and E) Real-time RT-PCR analysis was performed for the *E5* and *IFN*-β genes in HaCaT/E5 cells untreated or treated with dexamethasone (6, 12, or 24 h). Data were normalized as for panel A. Concentrations from dexamethasone-untreated HaCaT/E5 cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. **, $P < 0.01$; *, $P < 0.05$.

E5-expressing cells compared with control cells. Our results demonstrate that HPV16 E5 stimulates IRF-1 expression at a transcriptional level through both STAT1 and NF-κB consensus sequences in the IRF-1 promoter.

IRF-1 binds the IFN-β promoter and stimulates transcription in E5-expressing cells. We then investigated whether IRF-1 could directly regulate the *IFN*-β gene promoter activity in E5-expressing cells. The *in vivo* binding of IRF-1 to the *IFN*-β gene promoter was determined by ChIP analysis in both HaCaT and W12 cells (Fig. 5A). After DNA immunoprecipitation with specific IRF-1 Abs, real-time PCR amplification of the IRF-E binding site present on the *IFN*-β gene promoter/enhancer showed that the binding of IRF-1 was substantially increased upon 24 h of treatment in HaCaT/E5 cells compared

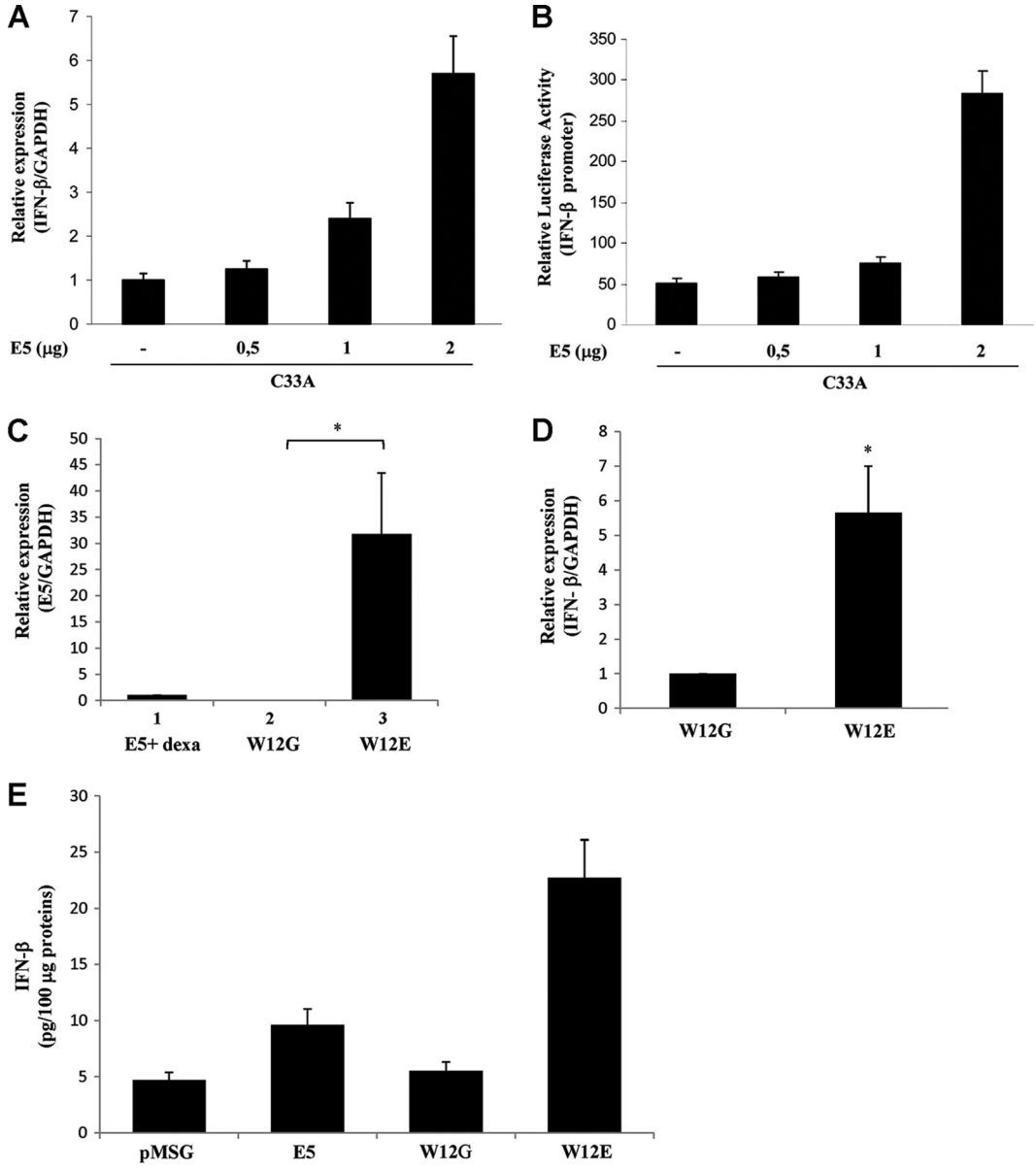

FIG. 2. HPV16 E5 induces IFN-β expression in C33A and W12 cells. (A) Real-time RT-PCR analysis was performed for the *IFN*-β gene in C33A cells transfected with increasing doses of E5-expressing vectors as indicated. Data were normalized as for Fig. 1A. (B) C33A cells were transfected with the IFN-β–luc reporter and increasing doses of E5 expression vector as shown in panel A. Luciferase activity was measured 24 h after transfection. Means ± standard deviations from two separate experiments were calculated after normalization with the pAct *Renilla* activity. (C) Real-time RT-PCR analysis was performed for the E5 gene in dexamethasone-treated HaCaT/E5 cells (lane 1) and in W12G and W12E cell clones (lanes 2 and 3). Data were normalized as for Fig. 1A. Levels from HaCaT/E5 cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. *, $P < 0.05$. (D) Real-time RT-PCR analysis was performed for the IFN-β gene in W12G and W12E cell clones. Data were normalized as for panel A. Levels from W12G cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. *, $P < 0.05$. (E) ELISA results for intracellular IFN-β. Total protein extracts (100 μg) from HaCaT/pMSG, HaCaT/E5, W12G, and W12E cells were used to determine the levels of IFN-β protein. Means ± standard deviations of three independent experiments are shown.

with HaCaT/pMSG cells (3-fold enrichment) and in W12E cells compared with W12G cells (18-fold enrichment).

This increase resulted in significant stimulation of IFN-β transcriptional activity in HaCaT/E5 cells (Fig. 5B, lane 5 versus lane 3 [~4-fold increase] and lane 5 versus lane 1 [13-fold increase]); these results were comparable with the increase induced by IRF-1 overexpression (Fig. 5B, lane 5 versus lane 4). Interestingly, when E5-expressing cells were cotransfected with the IRF-1-expressing vector, a synergicistic effect of E5 and IRF-1 on the *IFN*-β promoter stimulation was observed (Fig. 5B, lane 6 versus lanes 4 and 5).

To assess whether this effect was also operative for IRF-1-specific target genes, the transcriptional activity of a synthetic reporter construct, pISRE-TA, bearing five copies of consen-

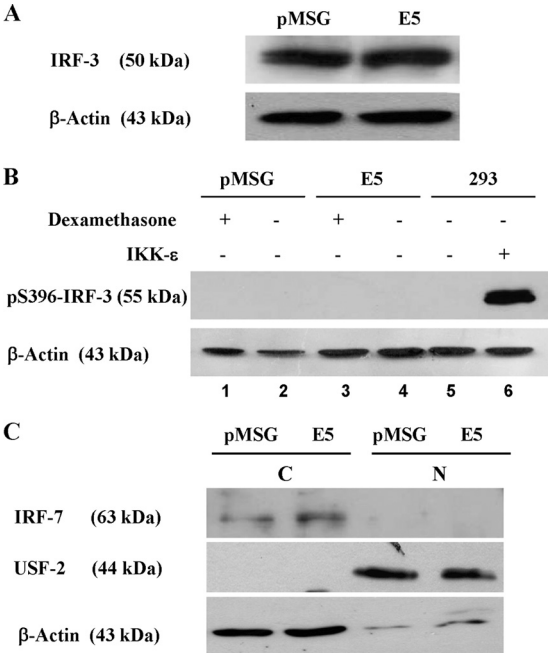

FIG. 3. HPV16 E5 does not modulate activity of IFN-β master regulators. Whole-cell extracts (50 μg) (A and B) or cytoplasmic and nuclear extracts (50 μg) (C) from dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells were analyzed by Western blotting with the specific antibodies anti-IRF-3 (A), anti-phospho-IRF-3 (B), and anti-IRF-7, anti-USF2, and anti-β-actin (C). In panel B, 293 cells transfected with IKK-ε were used as controls for IRF-3 activation. Representative data from three independent experiments are shown.

sus IRF-E sequences, was analyzed. As shown in Fig. 5C, the relative IRF-E-regulated luciferase activity was increased in E5-expressing cells compared with control cells (lane 5 versus lane 3 [3.5-fold increase] and lane 5 versus lane 1 [4.3-fold increase]), and this activity was further stimulated upon cotransfection with the IRF-1-expressing vector (Fig. 5C, lane 6 versus lanes 4 and 5).

Neutralization of type I IFN has no effects on E5-induced IRF-1 expression. To demonstrate that the pathway targeted by E5 protein is due to stimulation of IFN-β activation through IRF-1 and not the opposite, we analyzed IRF-1 expression in HaCaT/E5-expressing cells after IFN-α/β neutralization. As expected and as shown in Fig. 6A and B, in control cells not expressing E5 stimulation of IRF-1 expression induced by 24 h of IFN-β treatment (lane 1 versus lane 2 [3-fold increase]) was completely reversed by addition of IFN-α/β-neutralizing Abs (Fig. 6A, lane 3 versus lane 2). Conversely, IRF-1 stimulation induced by E5 protein was not affected by the addition of IFN-α/β-neutralizing antibodies (lane 5 versus lane 4). These results, while proving the ability of neutralizing antibodies to inhibit the autocrine/paracrine type I IFN-mediated intracellular signaling that results in IRF-1 expression, strongly suggest that stimulation of IRF-1 induced by E5 protein is not mediated by an initial production of type I IFN in E5-expressing cells.

Knockdown of IRF-1 expression in E5-expressing cells abolishes IFN-β expression. To further assess the E5-induced IRF-1-dependent IFN-β stimulation, HaCaT/E5 cells were transfected with a specific IRF-1-targeting siRNA, and IFN-β expression was measured by real-time RT-PCR. The efficacy of siRNA on IRF-1 expression was assessed by Western blot analysis, and the results showed that IRF-1 was significantly reduced (80%) in cells expressing IRF-1-specific siRNA compared with cells expressing luciferase siRNA (Fig. 7A, lane 4 versus lane 3). A real-time RT-PCR assay using total RNA isolated by siRNA-expressing HaCaT/E5 cells treated with dexamethasone for 24 h and harvested 48 h posttransfection indicated that IRF-1 knockdown completely abolished IFN-β expression induced by E5 protein (Fig. 7B, lane 4 versus lane 2). IFN-β expression was instead not affected in luciferase siRNA-transfected cells under the same conditions (lane 3).

HPV16 E5 stimulates ISG expression. To determine whether the regulation of IRF-1 by HPV16 E5 had an effect on host cell gene expression, we examined two IRF-1-targeted ISGs, PKR and caspase 8. Real-time RT-PCR data showed a significant increase of PKR and caspase 8 mRNA and protein accumulation in HaCaT cells expressing E5 compared with control cells (Fig. 8A and B and C and D, respectively). Induction of PKR and caspase 8 mRNA by E5 was also confirmed in W12 clones. As shown in Fig. 8E and F, substantially higher levels of PKR and caspase 8 were observed in E5-expressing W12E cells than in W12G cells.

DISCUSSION

There has been sustained interest in using IFNs and other immunomodulators to treat benign and premalignant HPV-associated lesions; however, inconsistent clinical outcomes have been observed (19, 24), and the mechanistic bases for these inconsistencies are not yet understood.

In the present study, we addressed the role of the HPV16 E5 oncoprotein, which is expressed only before viral integration but is often lost as lesions progress toward malignancy, in type I IFN signaling. Our data demonstrate that E5, *per se*, is able to induce IFN-β by a mechanism involving IRF-1. An IFN gene expression signature is similarly induced by E5.

E5, together with E6 and E7, is considered an HPV oncoprotein (52). The role of E6 and E7 in HPV-mediated cell transformation has been well established (15), and both proteins have been implicated in IFN host response inhibition (4, 36, 42). Similarly, the complete HPV31 genome has been shown to suppress some interferon-inducible genes in keratinocytes (8). Conversely, the role of E5 in HPV-mediated carcinogenesis is still poorly understood, and its impact on the IFN system has not been yet defined. Interestingly, while E6 and E7 are expressed throughout the course of the disease and are required for transformed phenotype maintenance, E5 gene is often lost as lesions progress toward malignancy (47). This suggests that E5 may play a critical role in the early phases of cell transformation before virus integration. Recently, in a proposed model of HPV16-induced cervical carcinoma, the loss of regulatory episomes appears to be a crucial step for selection of integrants and cancer progression. This event has been associated with a transient antiviral state inducible by type I IFN (21, 37). Our results, showing that HPV16 E5 stimulates IFN-β and ISGs, make this protein a good candidate as the trigger to the transition phase from premalignant to malignant lesions when episomal viral forms are lost and expression of integrants is deregulated.

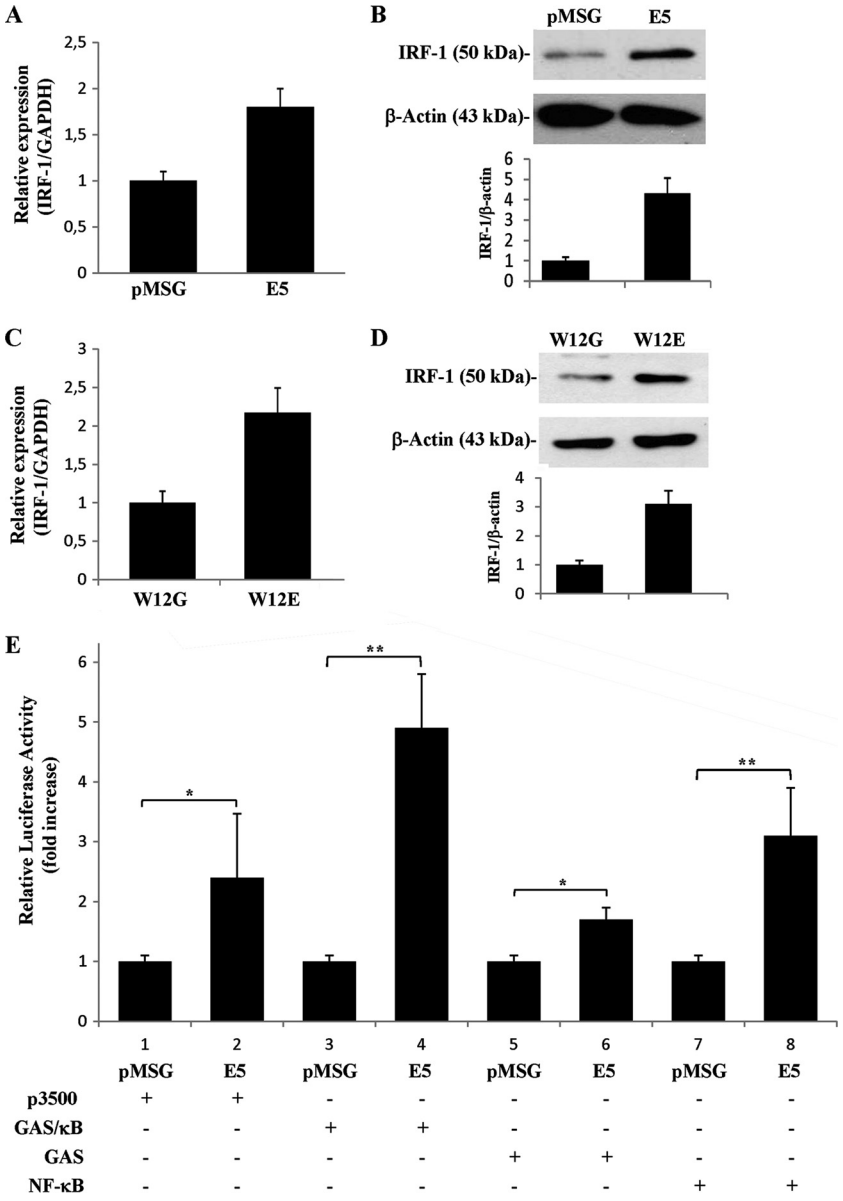

FIG. 4. HPV16 E5 induces IRF-1 expression. (A and C) Real-time RT-PCR analysis was performed for the IRF-1 gene in dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells (A) and in W12G and W12E cell clones (C). Data were normalized as for Fig. 1A, and levels from HaCaT/pMSG and W12G cells, respectively, were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. (B and D) Levels of IRF-1 protein were determined by Western blot analysis using specific anti-IRF-1 Abs. One representative experiment out of three performed is shown. IRF-1-specific bands were quantified by densitometry, and means ± standard deviations from three separate experiments were calculated and are reported as the IRF-1/β-actin ratio. (E) Dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells were transfected with p3500-luc, GAS/κB-luc, GAS-luc, and NF-κB-luc reporter constructs. Luciferase activity was measured 24 h after transfection. Means ± standard deviations from three separate experiments were calculated after normalization to the pAct *Renilla* activity. *, $P < 0.05$; **, $P < 0.01$.

Here we have also demonstrated that the increase in E5-mediated IFN-β expression is due to transcriptional activation. The induction of type I IFN is mainly regulated at the transcriptional level, wherein IRFs play central roles. Specifically, IRF-3 and IRF-7 have been implicated as main regulators of IFN gene transcription, with essential and distinct roles, and are responsible for prompt synthesis in response to infections. Indeed, IRF-3 is constitutively expressed in a variety of cell types, whereas IRF-7 is expressed at high levels only in plasmacytoid dendritic cells and is upregulated by IFN, lipopolysaccharide, and viral infection in most cell types. IRF-3 and IRF-7 are both activated by phosphorylation and then translocate into the nucleus, where they stimulate expression of target genes (22). We found that the E5 protein did not activate either IRF-3 or IRF-7. Given the paucity of cell lines suitable for *de novo* HPV infection, it is difficult to study different aspects of virus replication and IFN stimulation. We therefore cannot rule out that in the setting of HPV natural infection, some viral replication intermediates may signal through cytosolic receptors, as recently demonstrated for Epstein-Barr virus (1), and induce IFN by activating IRF-3 and/or IRF-7.

Nevertheless, our data indicate that, in the absence of this

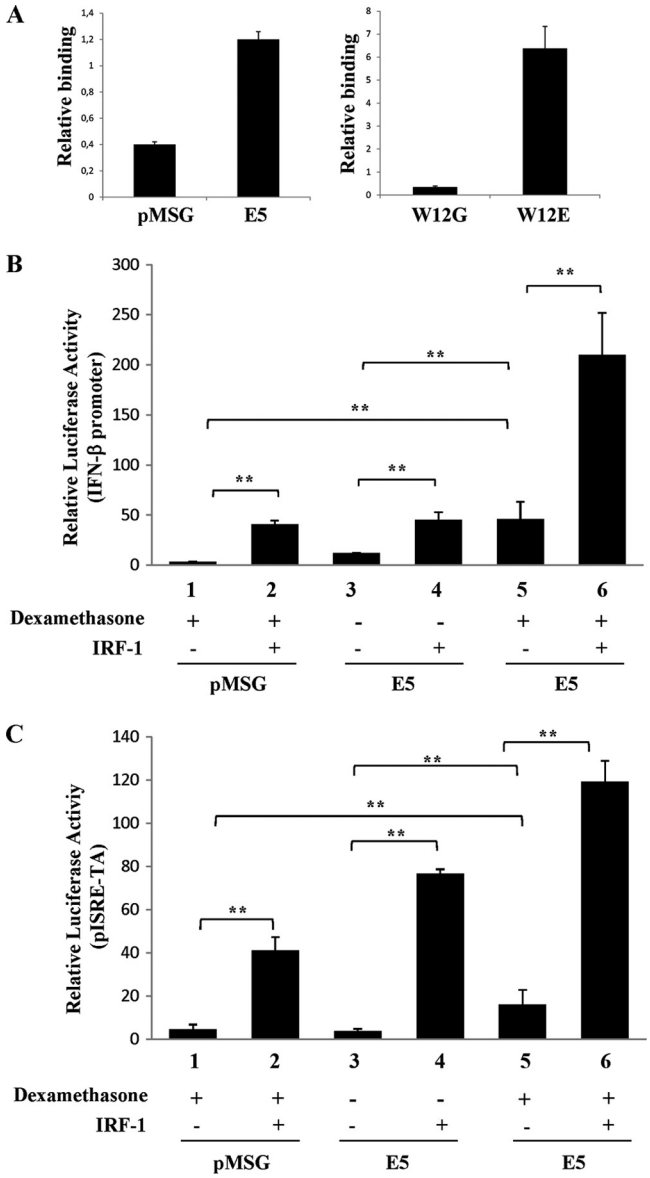

FIG. 5. IRF-1 binds the IFN-β promoter and stimulates transcription in E5-expressing cells. (A) Chromatin from dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells or W12G and W12E cell clones was immunoprecipitated using specific Abs against IRF-1. Anti-IgG Abs were used as the negative control. Quantification of binding of IRF-1-specific or IgG Abs was performed by real-time PCR using primers surrounding the IRF-E site on the *IFN*-β gene promoter; results shown were normalized as relative input. Data shown are means ± standard deviations of relative binding of replicates and are representative of three independent experiments. (B and C) HaCaT/E5 untreated cells (lanes 3 and 4) or dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells (lanes 1, 2, 5, and 6) were transfected with IFN-β–luc (B) or pISRE-TA-luc (C) reporter constructs and, where indicated, cells were cotransfected with an IRF-1-expressing vector (lanes 2, 4, and 6). Luciferase activity was measured 24 h after transfection. Means ± standard deviations from three separate experiments were calculated after normalization to the pAct *Renilla* activity. **, $P < 0.01$.

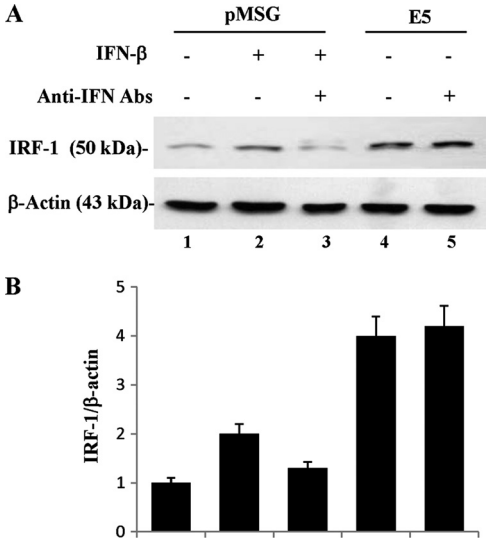

FIG. 6. Neutralizing anti-IFN-α/β antibodies have no effect on IRF-1 expression. (A) HaCaT/pMSG and HaCaT/E5 cells were treated with dexamethasone and, where indicated, 500 IU/ml of IFN-β for 24 h in the presence or absence of anti-IFN-α and anti-IFN-β (1,000 IU/ml). Whole-cell extracts were then analyzed by Western blotting with anti-IRF-1 and anti-β-actin Abs. Representative data from three independent experiments are shown. (B) IRF-1-specific bands were quantified by densitometry, and means ± standard deviations from three separate experiments were calculated and are reported as IRF-1/β-actin ratios.

classical activation pathway, E5 yet stimulates IFN-β gene transcription by means of IRF-1. On the other hand, the possibility that stimulation of IFN-β expression occurs independently from IRF-1 in E5-expressing cells and that IRF-1 stimulation is a consequence of IFN-β production was ruled out by IFN type I neutralization experiments that showed that the addition of IFN-α/β-neutralizing antibodies did not abolish IRF-1 stimulation induced by the E5 protein.

The casual relationship between IRF-1 stimulation and induction of IFN-β was definitely demonstrated in the experiments with IRF-1 silencing. Cells expressing specific IRF-1-targeting siRNA, in which the E5-mediated IRF-1 stimulation was inhibited by 80%, no longer produced IFN-β upon E5 expression. Nevertheless, since it is known that IFN-β in turn induces IRF-1, we can thus foresee that, in E5-expressing cells, an autocrine positive loop between IRF-1 and IFN-β does exist that leads to a substantial stimulation of an IFN signature.

At variance with IRF-3 and IRF-7, which are activated by phosphorylation, the different levels of IRF-1 expression are, indeed, one of the factors that dictate how it functions. Most cell types do not express IRF-1 at detectable levels, but its expression is rapidly induced at the transcriptional level following virus infection or exposure to various inducers, including IFNs and proinflammatory cytokines. We have shown that stimulation of IRF-1 expression by HPV16 E5 occurs through both the STAT1 and NF-κB consensus sequences on the *IRF-1* gene promoter. In agreement with these results, it has been reported that HPV16 E5 activates NF-κB (27). Conversely, so far no data on the modulation of STAT1 activity by HPV16 E5 have been reported. It will, therefore, be interesting to study the interference of this protein with signaling pathways triggered by IFN receptor engagement.

IRF-1 is a pleiotropic transcription factor that is critical for cell defense against viral infections but also crucial for the

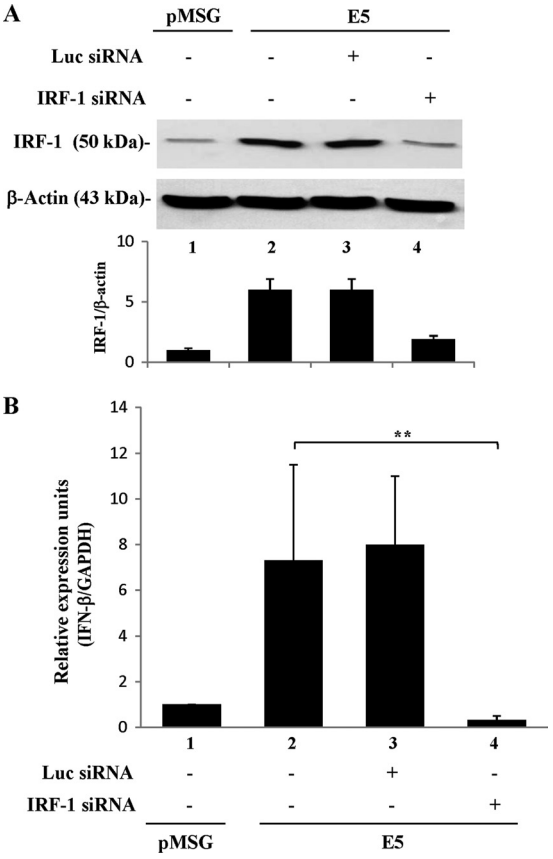

FIG. 7. Knockdown of IRF-1 expression in E5-expressing cells abolishes IFN-β stimulation. (A) IRF-1 expression was determined in whole-cell extracts from dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells expressing either IRF-1-targeting (IRF-1 siRNA) or control (Luc siRNA) siRNA by Western blotting with specific antibody against IRF-1. IRF-1-specific bands were quantified by densitometry, and means ± standard deviations from three separate experiments were calculated and are reported as IRF-1/β-actin ratios. (B) Real-time RT-PCR analysis was performed for the *IFN*-β gene in dexamethasone-treated HaCaT/pMSG cells (lane 1) and HaCaT/E5 cells (lanes 2, 3, and 4) expressing Luc siRNA (lane 3) or IRF-1 siRNA (lane 4). Data were normalized as for Fig. 1A, and levels from dexamethasone-treated HaCaT/pMSG cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. **, $P < 0.01$.

development of both the innate and adaptive immune responses (5, 30, 54). High IRF-1 expression levels are required for the induction of a set of target genes, including ISGs (20, 28). Notably, we have shown that E5 protein up-modulates IRF-1 expression at levels sufficient to stimulate ISG transcription. Indeed, in E5-expressing cells, IRF-1 upregulation in turn affects host gene expression mediated by IRF-E/ISRE.

Among the E5/IRF-1-stimulated genes, we identified PKR and caspase 8, specific targets of IRF-1 (28, 43). The role of PKR in the virus-induced antiviral response has been extensively demonstrated (16); however, recent reports also demonstrate that PKR is constitutively active in a variety of tumors and is required for tumor maintenance and growth (25).

Caspase 8 belongs to a family of proteases and plays a key role in apoptosis (14), but several nonapoptotic roles have been described, including promotion of cell motility, adhesion, and migration of cancer cells (31). Interestingly, caspase 8, together with caspase 10, has been also reported as an essential component that mediates NF-κB-dependent inflammatory responses in antiviral signaling (53).

Interestingly, chronic inflammation has been identified as a cofactor for HPV-driven cervical carcinogenesis (7), and IRF-1 is induced by and is an effector of inflammatory cytokines. This raises the possibility that IRF-1, as already reported for E5-induced NF-κB (27), can mediate an inflammatory response during HPV infection. E5, through IRF-1 upregulation, can thus affect host gene expression on a more global setting.

Notably, we also demonstrated the functional relationship between E5 expression, IRF-1, and IFN signature stimulation in the unique model of HPV16-related cervical squamous carcinogenesis W12 cells (23). This cell model has been extensively characterized, and it has been shown that it accurately resembles cervical neoplastic progression during long-term culture, with spontaneous transition from cells containing episomal HPV16 to a population containing only integrated HPV16 (2). It will therefore be interesting to assess whether the events that we have reported here are also seen in patients, based on analysis of IRF-1 expression and function during both active viral replication and selection of integrants. This could in part explain the various clinical outcomes of IFN therapy of papillomatoses and the differential regulation of HPV expression observed in various cancer cell lines upon IFN treatment (9, 19). Similarly, studies on the effect of E5 protein on IFN expression in the natural context of the complete viral genome during a *de novo* HPV infection would help to further elucidate its normal functions, as already reported for E5 activity on cell proliferation (40).

From our results, we can hypothesize the following scenario: during HPV productive episomal infection, stimulation of IRF-1 and IFN-β by E5 or other unchecked factors leads to the establishment of an antiviral state that, as reported, may accelerate episomal clearance (21, 37). As soon as only integrated virus is transcriptionally active, E5 is no longer expressed, and the IFN–IRF-1–mediated response is shut down. Meanwhile, high expression levels of the E6 and E7 oncogenes lead to inhibition of expression and/or activity of IFN synthesis key factors and the IFN-induced signal transduction pathway.

Modulation of IRF-1 expression by viral proteins has been reported for different viruses and can result either in inhibition of its expression, as reported for HPV E7 (36) and HCV core protein (10), or in up-modulation, as we demonstrated in the setting of HIV-1 infection (41, 44, 45) and here for HPV16 E5. The final output of this modulation, however, can be always regarded as a bright way of hijacking a key immune regulator to turn its activity to the virus's advantage.

In conclusion, we have presented data defining a critical role of the HPV16 E5 protein in IFN-β expression stimulation mediated by increased IRF-1 protein accumulation in human keratinocytes. An IFN gene expression signature was similarly induced. Our findings highlight an important function for the HR-HPV E5 oncogene and once more underline that great care should be taken during interferon treatment of HR-HPV-associated cervical lesions.

Inhibition of E5 and/or IRF-1 expression may also represent a new avenue for therapeutic interventions in early phases of infection, when activation of the innate immune

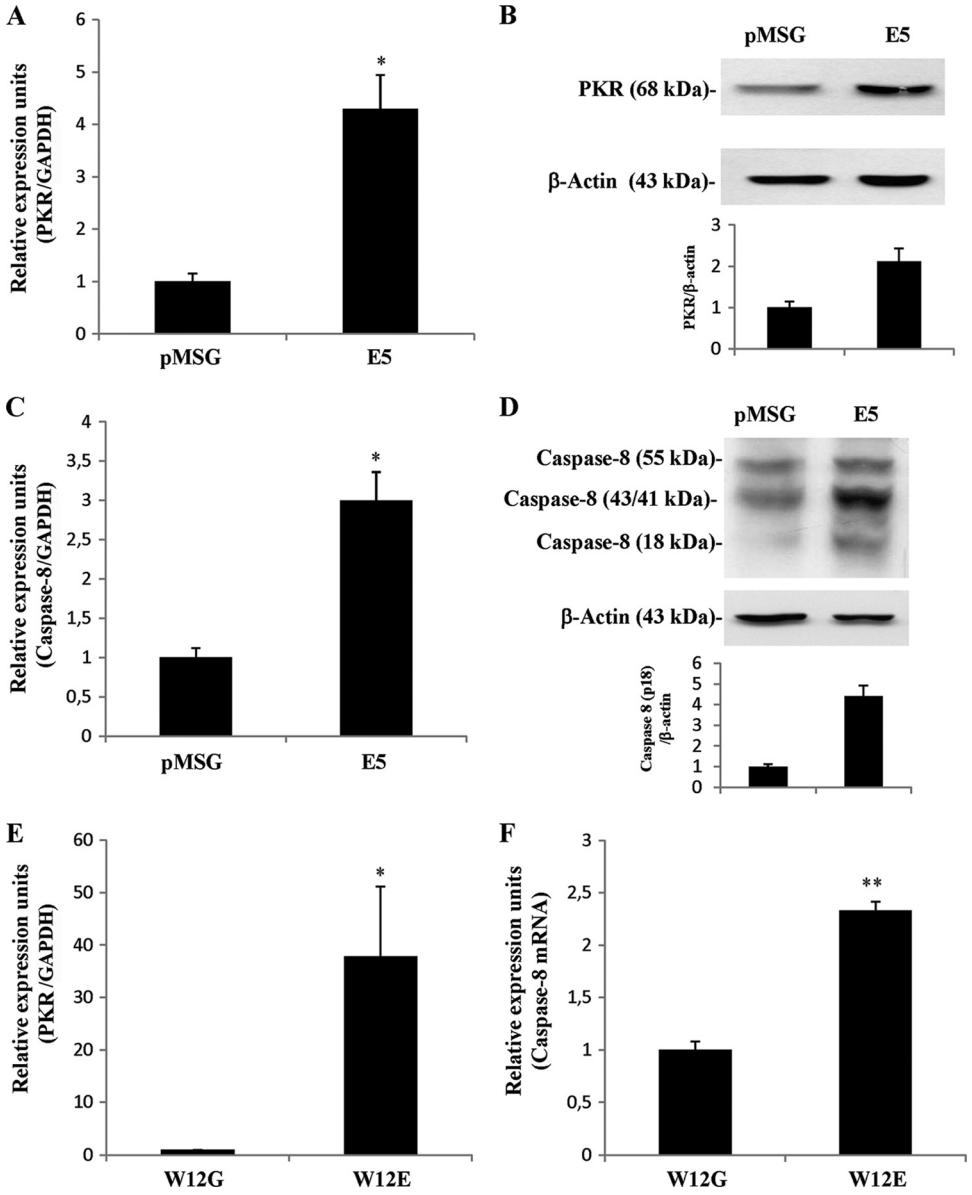

FIG. 8. HPV16 E5 induces expression of IRF-1 target genes. (A and C) Real-time RT-PCR analysis was performed for PKR (A) and caspase 8 (C) in dexamethasone-treated HaCaT/pMSG and HaCaT/E5 cells. Data were normalized as for Fig. 1A, and concentrations from HaCaT/pMSG cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. *, $P < 0.05$. (B and D) Levels of PKR and caspase 8 protein in both cell types were determined by Western blot analysis using specific anti-PKR and anti-caspase 8 antibodies recommended for detection of the p18 subunit and precursors of caspase 8, respectively. Representative data from three independent experiments are shown. PKR- and caspase 8-specific bands were quantified by densitometry, and means ± standard deviations from three separate experiments were calculated and are reported as PKR/β-actin or caspase 8/β-actin ratios. (E and F) Real-time RT-PCR analysis was performed for PKR and caspase 8 in W12G and W12E cells. Data were normalized as for Fig. 1A, and concentrations from W12G cells were set as the basis for the comparative results. Means ± standard deviations of three independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$.

response to protect may instead play an important role in disease progression.

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