Human papillomavirus type 16 E7 oncoprotein inhibits apoptosis mediated by nuclear insulin-like growth factor-binding protein-3 by enhancing its ubiquitin/proteasome-dependent degradation

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The E7 protein encoded by the oncogenic human papillomavirus type 16 has been shown to bind and inactivate insulin-like growth factor-binding protein-3 (IGFBP-3), the pro-apoptotic product of a tumour suppressor gene; however, the molecular mechanism underlying E7-induced inactivation of IGFBP-3 remains uncertain. In this study, we map the IGFBP-3-binding domain for E7 to the nuclear localization signal in the conserved C-terminal domain of IGFBP-3. Moreover, we demonstrate that both proteins interact in the nucleus and that E7 induces polyubiquitination and proteasome-dependent proteolysis of nuclear IGFBP-3 in cervical cancer cells. This leads to a dramatic shortening of the half-life of nuclear IGFBP-3, whereas the stability of an E7-non-binding IGFBP-3 mutant is not affected by E7. Finally, we show that E7-mediated destruction of nuclear IGFBP-3 correlates with the inhibition of IGFBP-3-induced apoptotic cell death. These data are consistent with E7-induced ubiquitin/proteasome-dependent inactivation of nuclear IGFBP-3.

Introduction

Human papillomaviruses (HPVs) of the high-risk type are associated with cervical squamous intra-epithelial lesions with a high potential for progression to invasive cervical squamous cell carcinoma in humans (1). HPV-16 is the most prevalent high-risk genotype. The viral E6 and E7 oncoproteins are necessary for maintenance of the transformed phenotype (1) and regularly expressed in HPV-positive cancers (2). According to current concepts of multi-step carcinogenesis, several growth-suppressing pathways must be independently subverted by E7 in order to immortalize primary human epithelial cells (3). In accordance with this hypothesis, E7 has the ability to interact with and functionally inactivate several cellular growth regulatory proteins, as best studied for the retinoblastoma tumour suppressor protein.

Insulin-like growth factor-binding protein-3 (IGFBP-3) is a key component of the insulin-like growth factor-I (IGF-I)/IGF-axis of growth regulatory proteins, which plays an important role in tumorigenesis (4). IGFBP-3 can bind IGF-I and thereby regulate the mitogenic activity of IGF-I in the extracellular environment. The IGFBP-3 gene is transcriptionally activated by the tumour suppressor p53 (5) and increased expression of IGFBP-3 contributes to both p53-dependent and -independent apoptosis (6). IGF/IGF-receptor (IGF-R)-independent actions of IGFBP-3 contribute also to its anti-proliferative and pro-apoptotic activities (7). For example, IGFBP-3 can induce programmed cell death in immortalized IGF-R-negative mouse fibroblasts (6), and IGFBP-3 mutants that do not bind IGFs can stimulate apoptosis in prostate cancer (8) and breast cancer cells (9). Moreover, the expression of an IGF-non-binding IGFBP-3 mutant induces apoptosis and inhibits prostate tumour progression in a transgenic mouse model of prostate cancer (10). The mechanisms underlying IGF-independent actions of IGFBP-3 may involve regulation of gene expression via activation of cell-surface receptor kinases or directly by nuclear IGFBP-3 (4). IGFBP-3 contains a nuclear localization sequence (NLS) in its conserved C-terminal domain (11), and importin-β-dependent import from the cytosol into the nucleus has been demonstrated (12). Moreover, after addition to the cell culture supernatants, IGFBP-3 has been detected in the nuclei of different human cancer cell types (8) and human keratinocytes (13). The interaction of IGFBP-3 with the nuclear retinoid X receptor was found essential for mediating the effects of IGFBP-3-induced apoptosis in prostate cancer cells (14), supporting the notion that nuclear IGFBP-3 functions in the induction of programmed cell death. Furthermore, it was shown that the abundance and pro-apoptotic activity of nuclear IGFBP-3 are regulated by ubiquitin/proteasome-dependent proteolysis (15). However, the nuclear functions of IGFBP-3 and the mechanisms that control the activity of nuclear IGFBP-3 are still poorly understood.

It was previously demonstrated that HPV-16 E7 can inhibit IGFBP-3-mediated apoptosis (16); the viral oncoprotein interacts with IGFBP-3 and triggers its proteolytic cleavage. Although these findings suggest that inactivation of IGFBP-3 is important for the oncogenic activity of E7, the precise mechanism underlying E7-dependent inactivation of IGFBP-3 remains uncertain. The aim of this study was to investigate functional consequences of the E7-IGFBP-3 interaction.

Material and methods

Plasmid constructions

The plasmids pcXIGFBP-3, pcXIGFBP-3KED253-255RGD, pcXIGFBP-3KGRKR228-232MDGEA, pcXls-IGFBP-3, pcXls-IGFBP-3KGRKR228-232MDGEA, pcXls-IGFBP-3A185-264, pcXneobHsE-Als-IGFBP-3 and pcXneobHsE-Als-IGFBP-3KGRKR228-232MDGEA are described (15). The plasmids pcXIGFBP-3A189-184 and pcXIGFBP-3A189-264 are derived from pS2F09 and pS2F09 (17). To generate pcXls-IGFBP-3A1-164, an EcoRI/XhoI IGFBP-3(165–264) fragment was isolated from pG4-SIGFBP-3gst-264 (Boris Mannhardt, Deutsches Krebsforschungszentrum, Heidelberg) and inserted into pX. The oligonucleotide 5′-aattcgatatcatgcagcgggcgcgacccacgctctgggccgctg-3′ and leader sequence was inserted into the EcoRI site. pGEX-4T1, pGST-HPV-16 E7 and pXHPV-16 E7 are described (16). The retroviral expression vector pSITVneo-HPV-16 E7 was generated by insertion of HPV-16 E7 cDNA as NotI/EcoRI fragment into pSITVneo M2-ires-TRSID-ires-Puro self-inactivating retrovirus vector, containing the tetracycline-transactivator/represor sequences (18). Cells were

Abbreviations: GST, glutathione S-transferase; HPV, human papillomavirus; IGF-I, insulin-like growth factor-I; IGFBP-3, insulin-like growth factor-binding protein-3; IGF-R, IGF-receptor; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; pKB, retinoblastoma protein; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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selected for stable transfectants by puromycin and single clones infected with the pSITVneo-HPV-16 E7 self-inactivating retrovirus vector. Stable transfectants were selected by neomycin and tested for HPV-16 E7 expression upon induction by 1 µg/ml doxycycline.

GST pull-down assays

Glutathione S-transferase (GST) pull-down assays were performed as described (16) using conditioned media from U-2OS cells secreting ectopically expressed IGFBP-3s (20).

Co-immunoprecipitation

Cells were incubated with 100 µM LLLN (Sigma, Vienna) for 3 h and co-immunoprecipitation was conducted as described (16), using goat anti-HPV-16 E7 (21), goat anti-IGFBP-3 antibodies (15) or goat control serum.

Indirect immunofluorescence

Cells were fixed with 4% (wt/vols) paraformaldehyde/phosphate-buffered saline (PBS) and permeabilized with 0.1% (wt/vols) Na-citrate/0.3% (vol/vols) Triton-X-100. After blocking with 1% (wt/vols) bovine serum albumin/PBS, cells were incubated for 1 h with goat polyclonal anti-IGFBP-3 antibodies (DSL, Sinsheim) in blocking buffer. Cells were washed in 1% bovine serum albumin/PBS, blocked with 1% (wt/vols) bovine serum albumin/PBS and incubated for 1 h at room temperature with affinity-purified rabbit polyclonal anti-HPV-16 E7 antibodies (20 µg/ml), which were pre-incubated with 2.5 µg/µl of the 20,000g supernatant of an NIH3T3 total-cell extract in blocking buffer for 1 h at room temperature. Cells were processed for immunofluorescence analysis as described (2) and viewed using confocal microscopy.

Subcellular fractionation

Cell fractions were conducted using the Qproteome Cell Compartment Kit (Qiagen). IGFBP-3 and HPV-16 E7 abundance was analysed by western blotting in cytosolic, membranous and nuclear fractions produced from 5 × 10^6 cells. Antibodies used as cell fractionation markers are as follows: α-M2PK (Schebo Biotech AG, Wettenberg), α-GM130 and α-Calnexin (BD-Biosciences, Schwechat) and α-Lamin B (Oncogene, Boston, MA).

Western blotting

Western blotting was performed as described (15).

Protein half-life determination

C33AdoxE7 cells were transfected with plasmids expressing Δls-IGFBP-3 or Δls-IGFBP-3KGRKR228-232MDGEA together with a β-galactosidase expression vector to monitor equal transfection efficiency and loading. E7 expression was induced with doxycycline, and IGFBP-3 half-lives were determined as described (15).

In vivo ubiquitination assay

C33A and U-2OS cells were transiently cotransfected with expression vectors for His6-Δls-IGFBP-3, His6-Δls-IGFBP-3KGRKR228-232MDGEA, HPV-16 E7, empty vector and pX-Flag-CMV-10-Ubiquitin as indicated. Twenty-four hours later, cells were incubated with 100 µM LLLN for 3 h and lysed in stabilizing buffer [1% (wt/vols) sodium dodecyl sulphate, 50 mM Tris–HCl, pH 6.8], sonified and denatured for 10 min at 95°C. The His-tag-IGFBP-3s were purified as described (15), separated on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected by western blotting using polyclonal goat anti-IGFBP-3 and monoclonal anti-Flag antibodies (Sigma).

In vitro ubiquitination assay

Secreted His-tag-IGFBP-3 wild-type protein was purified from supernatants of U-2OS cells expressing IgK-His-Δls-IGFBP-3 (15). HPV-16 E7 protein was purified as described (20). Adenosine triphosphate-dependent ubiquitin conjugation of IGFBP-3 (+/− HPV-16 E7) was conducted using ubiquitin: GST-ubiquitin mix (+/-1), ubiquitin-vinyl-sulphone and Ubiquitin Protein Conjugation Kit (BostonBiochem, Cambridge, MA).

Apoptosis assay

C33A cells were transfected with pEGFP C1, pXHPV-16 E7, empty vector and pXIGFBP-3 vectors as indicated. After medium change, the cells were cultured 48 h in Dulbecco’s modified Eagle’s medium/10% foetal calf serum, trypsinized, collected and washed with 2 ml PBS. Cells were stained in 50 µl annexin-V-binding buffer/2 µl annexin-V-Alexa Fluor 647 conjugate/5 µM Sytox Orange (Invitrogen, Lofer) for 15 min. After adding 150 µl 1 × annexin-V-binding buffer, cells were centrifuged and resuspended in 500 µl annexin-V-binding buffer and analysed by flow cytometry using compensated detection settings. Cells were gated for positive transfection by enhanced green fluorescent protein. To rule out necrosis and non-apoptotic cell death, enhanced green fluorescent protein-positive cells were gated into a Sytox Orange-positive (cells with permeabilized membrane) and a Sytox Orange-negative (cells with intact membrane) fraction. Apoptotic cells were determined in the Sytox Orange-negative cell populations by annexin-V-Alexa Fluor 647 staining.

Statistical analysis

Student’s t-test was used.

Results

HPV-16 E7 binds to the NLS in the conserved C-terminal domain of IGFBP-3

HPV-16 E7 interacts with IGFBP-3 via a domain localized within its C-terminal region (16). To identify the E7-binding domain in IGFBP-3, we employed IGFBP-3 mutants (Figure 1A). Each mutation targets a distinct region of IGFBP-3, the conserved N-terminal domain, the non-conserved central and the conserved C-terminal domain (IGFBP-3Δ89-264, Δ185-264, Δ89-184, Δ11-164, KGRKR228-232MDGEA and KED253-255RGD), respectively. IGFBP-3 wild-type and the mutants were expressed in human U-2OS cells, and the secreted IGFBP-3 proteins analysed for their interaction with E7 in GST pull-down experiments. IGFBP-3 wild-type interacted strongly and in a dose-dependent manner with purified GST-16 E7 but not with GST alone (Figure 1B). While the interaction with E7 was completely abolished in constructs lacking the C-terminal domain of IGFBP-3 (IGFBP-3Δ89-264 and IGFBP-3Δ1185-264), IGFBP-3Δ89-184 (containing the conserved N-terminal and C-terminal regions) and IGFBP-3Δ1-164 (containing the conserved C-terminal domain) were able to bind E7, although with reduced efficiency compared with IGFBP-3 wild-type (Figure 1C). These observations indicate that E7 binds to the conserved C-terminal domain of IGFBP-3. Two additional IGFBP-3 mutants were employed to precisely map the domain of IGFBP-3 required for the interaction with E7, IGFBP-3KGRKR228-232MDGEA in which the NLS is inactivated and IGFBP-3KED253-255RGD in which three amino acids in the NLS-adjacent region are altered (KED to RGD) (17). The binding of E7 to IGFBP-3 was completely abrogated by the mutation of the NLS, whereas the mutation of the KED motif had no effect on binding (Figure 1D). These findings indicate that the NLS in the C-terminus of IGFBP-3 is required for its interaction with E7.

HPV-16 E7 co-localizes with IGFBP-3 in the nucleus and induces a reduction in the nuclear IGFBP-3 steady-state levels

To determine a potential nuclear interaction between both proteins, U-2OS cells were transiently transfected with pXAlS-IGFBP-3 encoding a form of IGFBP-3 that localizes predominantly to the nucleus (15). The cells were stained with α-IGFBP-3 antibodies and viewed by indirect immunofluorescence microscopy using a confocal laser scanning system. The anti-IGFBP-3 antibodies predominantly stained the nucleus of the transiently transfected cells (Figure 2A, panel 1, top). In U-2OS cells transiently transfected with pXAlS-IGFBP-3KGRKR228-232MDGEA, the anti-IGFBP-3 antibodies predominantly stained the cytosol (Figure 2A, panel 1, middle). No signal was obtained with mock-transfected cells (Figure 2A, panel 1, bottom). In U-2OS cells transiently transfected with pXHPV-16 E7, a predominantly nuclear staining was detected by antibodies to HPV-16 E7 (Figure 2A, panel 2). In U-2OS cells transiently expressing both nuclear IGFBP-3 and HPV-16 E7, anti-IGFBP-3 (red) and anti-HPV-16 E7 (green) antibodies revealed a similar staining pattern in the nucleus (Figure 2A, panels 3 and 5, top); however, in cells co-expressing E7 with nuclear IGFBP-3, the intensity of IGFBP-3 staining was strongly reduced (Figure 2A, panel 3, top), whereas co-expression of IGFBP-3 did not affect the intensity of E7 staining (Figure 2A, panel 5, top). These findings suggest that co-expression of E7 and IGFBP-3 in the nucleus leads to a drastic reduction of the IGFBP-3 level. When U-2OS cells were transiently cotransfected with pXAlS-IGFBP-3KGRKR228-232MDGEA and pXHPV-16 E7, the IGFBP-3 staining was predominantly cytosolic and E7 staining predominantly nuclear.
The staining intensity of the Dls-IGFBP-3KGRKR228-232MDGEA protein was not significantly affected by E7 cotransfection, suggesting that the abundance of the cytosolic E7-non-binding IGFBP-3 mutant is not reduced by E7-co-expression. To support these conclusions, U-2OS cells were transiently transfected with pX Dls-IGFBP-3 and increasing amounts of pXHPV-16 E7 and the impact of E7 on the nuclear IGFBP-3 levels monitored by western blotting (Figure 2B). This experiment was three times repeated and the IGFBP-3 bands were densitometrically analysed. E7 induced a significant reduction in the steady-state levels of nuclear IGFBP-3 (Figure 2B, left). The steady-state levels of the predominantly cytosolic E7-non-binding mutant Δls-IGFBP-3KGRKR228-232MDGEA were not altered by co-expression of E7 (Figure 2B, right). These findings indicate that the abundance of nuclear IGFBP-3 protein is reduced by co-expression of HPV-16 E7.

Since HPV-16 cause cervical cancer (1), we wondered whether the E7-induced reduction in nuclear IGFBP-3 protein level is caused by proteasome-dependent proteolysis in cervical cancer cells. To investigate this, we performed a fractionation experiment analysing endogenous IGFBP-3 protein levels in subcellular fractions of epoxomicin

(Figure 2A, panels 3 and 5, bottom). The staining intensity of the Δls-IGFBP-3KGRKR228-232MDGEA protein was not significantly affected by E7 cotransfection, suggesting that the abundance of the cytosolic E7-non-binding IGFBP-3 mutant is not reduced by E7-co-expression. To support these conclusions, U-2OS cells were transiently transfected with pXΔls-IGFBP-3 and increasing amounts of pXHPV-16 E7 and the impact of E7 on the nuclear IGFBP-3 levels monitored by western blotting (Figure 2B). This experiment was three times repeated and the IGFBP-3 bands were densitometrically analysed. E7 induced a significant reduction in the steady-state levels of nuclear IGFBP-3 (Figure 2B, left). The steady-state levels of the predominantly cytosolic E7-non-binding mutant Δls-IGFBP-3KGRKR228-232MDGEA were not altered by co-expression of E7 (Figure 2B, right). These findings indicate that the abundance of nuclear IGFBP-3 protein is reduced by co-expression of HPV-16 E7.

Since HPV-16 cause cervical cancer (1), we monitored the intracellular distribution of endogenous E7 and IGFBP-3 in the HPV-16-positive cervical carcinoma CaSiKi cells by indirect immunofluorescence experiments using confocal laser scanning microscopy. When CaSiKi cells were stained with the anti-HPV-16 E7 and anti-IGFBP-3 antibodies, predominantly cytoplasmic staining was found for IGFBP-3 (Figure 2C, upper panel, green), whereas in most of the cells, the E7 staining was nuclear and cytoplasmic (Figure 2C, upper panel, red). When both signals were superimposed, we found a co-localization of E7 and IGFBP-3 on distinct areas around the nuclear envelope (Figure 2C, upper panel, merge). The pre-immunesera did recognize neither IGFBP-3 nor E7 in CaSiKi cells (2,15 and data not shown). These results suggest that endogenous epoxomicin-stabilized IGFBP-3 and E7 co-localize near the nuclear envelope or the nuclear periphery in CaSiKi cells and that E7 induces a reduction of the IGFBP-3 protein levels at these sites.

**HPV-16 E7-induced reduction of nuclear IGFBP-3 protein level requires a functional proteasome**

Since nuclear IGFBP-3 is a target for ubiquitin/proteasome-dependent destruction in U-2OS cells (15), we wondered whether the E7-induced reduction in nuclear IGFBP-3 protein level is caused by proteasome-dependent proteolysis in cervical cancer cells. To investigate this, we performed a fractionation experiment analysing endogenous IGFBP-3 wild-type and E7 protein levels in subcellular fractions of epoxomicin
and mock-treated CaSki cells. We prepared nuclear, cytosolic and membranous fractions and analysed the level of endogenous IGFBP-3 and E7 proteins by western blotting (Figure 3A). As expected, the nuclear protein lamin B was retrieved in the nuclear fraction, whereas the cytosolic marker pyruvate kinase (M2-PK) was exclusively detected in the cytosol fraction, and the Golgi marker GM130 and the endoplasmic reticulum-specific protein calnexin were found exclusively in the membrane fraction, suggesting that a clean separation of proteins from the nuclear, cytosolic and membranous fractions had been achieved. In the mock-treated CaSki cells, the majority of the endogenous IGFBP-3 wild-type protein was detected in the membrane fraction but a significant amount was also localized in the nuclear fraction, indicating that endogenous IGFBP-3 exists in the nucleus as well as in the cytoplasm of CaSki cells. E7, which is well described as a nuclear and cytoplasmic protein (2,3,23), was detectable in cytoplasmic as well as nuclear CaSki cell fractions (Figure 3A), as shown before (24). Though E7 is predominantly localized in nuclei (Figure 2C), little E7 protein could be fractionated in the nuclear fraction (Figure 3A). This can be explained by leakage, which can happen especially for small molecules like the 11 kda E7 protein. Thus, these findings underline that significant amounts of both IGFBP-3 and E7 protein are co-localized in the nucleus as shown in Figure 2. Epoxomicin treatment led to a significant increase of the IGFBP-3 protein stability (Figure 3C, compare lanes 5 and 6), thus linking the reduction of the IGFBP-3 protein level analysed by western blotting using anti-IGFBP-3 antibodies (Figure 4B, upper panel).

To present additional evidence that nuclear IGFBP-3 is stabilized by epoxomicin in E7-expressing cervical cancer cells, epoxomicin was added to C33A cells transiently transfected with pXAs-IGFBP-3 and/or pXHPV-16 E7. Nuclear localization of both proteins was controlled by indirect immunofluorescence analysis (Figure 3B). Both proteins were expressed in the nucleus and co-expression of E7 and IGFBP-3 led to reduced nuclear IGFBP-3 level in C33A cells. Lysates from these cells were separated on a SDS–PAGE and the IGFBP-3 protein level analysed by western blotting using anti-IGFBP-3 antibodies (Figure 3C). In this experiment, E7 induced a strong reduction in the steady-state levels of Δls-IGFBP-3 (Figure 3C, compare lanes 1 and 3). The abundance of Δls-IGFBP-3 was not reduced by E7 in epoxomicin-treated cells, although epoxomicin treatment led to increased E7 protein levels, indicating that E7-induced degradation of IGFBP-3 occurs via proteasome-dependent proteolysis (Figure 3C, compare lane 4 with lanes 1–3). The steady-state levels of the E7-non-binding mutant Δls-IGFBP-3-3KGRKR28-232MDGEA, which is predominantly localized in the cytosol of C33A cells (Figure 3B), were not significantly altered by E7 (Figure 3C, compare lanes 5 and 6), thus linking the reduction of the IGFBP-3 steady-state levels in the nuclei with its binding to E7. Together, these experiments indicate that E7 induces proteasome-dependent destruction of nuclear IGFBP-3.

E7-dependent reduction of nuclear IGFBP-3 is caused by decreased protein stability

To analyse the mechanism underlying E7-induced reduction of Δls-IGFBP-3, the half-life of Δls-IGFBP-3 was determined in the presence and absence of E7 (Figure 4). To do this, C33A/AdoxE7 cells were employed in which E7 is expressed under the control of a doxycycline-inducible promoter. E7 was well expressed 15 h after induction with doxycycline but not detectable without doxycycline (Figure 4A), indicating that E7 expression is tightly regulated. To determine the influence of E7 on the half-life of nuclear IGFBP-3, C33A/AdoxE7 cells were transiently transfected with pXAs-IGFBP-3 and E7 expression was induced. Cycloheximide was added to block cellular protein synthesis, ruling out any influence of altered protein synthesis on the IGFBP-3 steady-state levels and metabolic half-life. Cells were lysed in sodium dodecyl sulphate sample buffer following a time course and the IGFBP-3 levels analysed by SDS–PAGE and western blotting using polyclonal anti-IGFBP-3 antibodies (Figure 4B, upper panel). The intensity of the IGFBP-3 bands was measured densitometrically and the half-life (T1/2) calculated by half-logarithmic plotting of the intensity of the bands against the cycloheximide incubation time (Figure 4B, lower panel). The half-life of Δls-IGFBP-3 was 94.1 ± 20.3 (x ± SD, n = 4) min in the absence of E7 and 37.3 ± 3.3 (x ± SD, n = 4) min without E7 and 91.2 ± 30.3 (x ± SD, n = 4) min (not significant, +E7 versus −E7) min in the presence of E7. The same assay performed with predominantly cytosolic non-E7-binding IGFBP-3 (Δls-IGFBP-3-3KGRKR228-232MDGEA) showed a half-life of 70.0 ± 36.9 (x ± SD, n = 3) min without E7 and 91.2 ± 30.3 (x ± SD, n = 4) min (not significant, +E7 versus −E7) min with E7 (Figure 4C, lower panel). In conclusion, these findings demonstrate that E7 efficiently induces the proteolysis of Δls-IGFBP-3.

Identifying Δls-IGFBP-3 as E7 target, we aimed to strengthen this finding by the demonstration that both proteins interact in a cellular context using a co-immunoprecipitation assay. To do this, U-2OS cells, which display an intrinsic high transfection efficiency, were transiently cotransfected with pXHPV-16 E7 and/or pXAs-IGFBP-3 as indicated (Figure 4D), and the 26S proteasome inhibitor LLNl was added 3 h before cell lysis. Goat anti-IGFBP-3 antibodies specifically co-precipitated E7 with IGFBP-3 from extracts of cells co-expressing E7 and Δls-IGFBP-3 (Figure 4D), and goat anti-E7 antibodies specifically co-precipitated Δls-IGFBP-3 with E7 from extracts of cells co-expressing E7 and nuclear IGFBP-3, whereas immunoprecipitation of IGFBP-3 was not achieved from the control cells (Figure 4D). Moreover, goat pre-immunserum did precipitate neither IGFBP-3 nor E7 in extracts from IGFBP-3- and E7-expressing cells (Figure 4D). These data suggest that E7 can form a specific complex with Δls-IGFBP-3 in a cellular context.

HPV-16 E7 enhances polyubiquitination of nuclear IGFBP-3

Subsequently, an in vivo ubiquitination assay was performed to test whether E7 triggers IGFBP-3 proteolysis by the ubiquitin/proteasome pathway (Figure 5A). U-2OS cells were transiently cotransfected with expression vectors for nuclear His-tag-IGFBP-3 and 3× Flag-tag-ubiquitin and lysates prepared 24 h later. His-tag-Δls-IGFBP-3 derivatives were purified, normalized for the presence of identical amounts of IGFBP-3 by western blotting, using anti-IGFBP-3 polyclonal sera (Figure 5A, lower panels), and analysed for polyubiquitination by western blotting using anti-Flag antibodies (Figure 5A, lane 1, upper panel). Under these conditions, polyubiquitinated IGFBP-3 conjugates could be detected. When E7 was transiently co-expressed (Figure 5A, lane 2), a significant increase of the amount of polyubiquitinated IGFBP-3 was detected (Figure 5A, lane 2, upper panel). Similar experiments were performed in C33A cervical cancer cells. Again, the level of polyubiquitinated Δls-IGFBP-3 was higher with E7 as compared to E7 without Δls-IGFBP-3 in the absence of E7 (Figure 4A).

Fig. 2. Co-localization of HPV-16 E7 and IGFBP-3 in U-2OS and CaSki cells. U-2OS cells were transiently cotransfected with empty vector and expression vectors for predominantly nuclear IGFBP-3 (pXAs-IGFBP-3), predominantly cytosolic IGFBP-3 (pXAs-IGFBP-3-3KGRKR228-232MDGEA) and pXHPV-16 E7, as indicated and analysed by indirect immunofluorescence microscopy and western blotting. (A) Cells were stained with anti-IGFBP-3 (red) and anti-HPV-16 E7 (green) antibodies and the DNA stain 4′, 6-diamidino-2-phenylindole (blue). (B) Nuclear and cytosolic IGFBP-3 levels in E7 co-expressing U-2OS cells monitored by western blotting using polyclonal anti-IGFBP-3 and HPV-16 E7 antibodies. M2-PK served as an input control (** P < 0.001). (C) HPV-16-positive CaSki cells were processed for indirect immunofluorescence microscopy to detect endogenous E7 and IGFBP-3. Cells were incubated for 3 h with dimethyl sulfoxide (upper panels) or 10 μM epoxomicin (lower panels), fixed and stained with anti-IGFBP-3 (green) and anti-HPV-16 E7 (red) antibodies, as indicated. (A, C) Cells were viewed using a confocal scanning system.
expression plasmids for predominantly nuclear IGFBP-3 (pXls-IGFBP-3) and HPV-16 E7. A western blot was performed using antibodies against Laemmli sample buffer. Samples were separated by SDS–PAGE and or dimethyl sulfoxide for 3 h and harvested in sodium dodecyl sulphate–indicated.

IGFBP-3 and HPV-16 E7 levels were analysed in total cell lysates, cytosolic, indicated, and subjected to lysis and cell fractionation. The endogenous IGFBP-3 was transiently expressed in C33A cells under the same conditions, we found a significant increase in the percentage of cells undergoing apoptosis, suggesting that predominantly nuclear IGFBP-3 can read-

HPV-16 E7 inhibits apoptotic cell death induced by nuclear IGFBP-3 in C33A cells

Transient transfection of C33A cells with an expression vector for IGFBP-3 wild-type induced a significant increase in the percentage of annexin-V-positive cells relative to mock-transfected cells (Figure 6), suggesting that transiently expressed IGFBP-3 wild-type induces apoptosis in these cells. Co-expression of E7 with IGFBP-3 led to a significant reduction in the amount of apoptotic C33A cells, indicating that E7 can abrogate IGFBP-3 wild-type-induced apoptosis in C33A cells, as shown previously in PC3 cells (16). When Δls-IGFBP-3 was transiently expressed in C33A cells under the same conditions, we found a significant increase in the percentage of cells undergoing apoptosis, suggesting that predominantly nuclear IGFBP-3 can readily induce apoptosis in the C33A cells. Co-expression of E7 resulted in a significant abrogation of the pro-apoptotic activity of Δls-IGFBP-3 (Figure 6). Moreover, we found that transient expression of the predominantly cytosolic E7-non-binding mutant Δls-IGFBP-3GRKR228-232MDGEA led also to a significant increase in the percentage of apoptotic cells (Figure 6); however, co-expression of E7 resulted not in a significant abrogation of the pro-apoptotic activity of this IGFBP-3 mutant (Figure 6). Together, these findings suggest that E7 specifically prevents apoptosis mediated by nuclear IGFBP-3.

Discussion

In this study, we demonstrate for the first time that the HPV-16 E7 oncoprotein regulates the abundance of nuclear IGFBP-3 by inducing its polyubiquitination and proteasome-dependent degradation in cervical cancer cells. This contributes to the inhibition of apoptotic cell death induced by nuclear IGFBP-3, suggesting that the accumulation of nuclear IGFBP-3 is not compatible with cervical cancer cell survival. These results contribute to a better understanding of the role of ubiquitin/proteasome-dependent proteolysis for the oncogenic activity of E7, and are consistent with the model that E7 needs to inactivate several tumour suppressor pathways important to the oncogenic potential of the virus (3).

A nuclear interaction with HPV-16 E7 triggers polyubiquitination and degradation of IGFBP-3

The ability to interact with E7 is severely impaired by a point mutation in the NLS localized in the conserved C-terminal domain of IGFBP-3. Since none of the deletion mutants employed in this study had the capability to bind E7 with high affinity, the intact structure of IGFBP-3, including both N-terminal and C-terminal domains, may be required for efficient E7 binding, as was shown for high-affinity binding of IGFBP-3 to IGF-I (25). IGFBP-3 has been shown to bind via its NLS to importin β, and nuclear import of IGFBP-3 is mediated in a Ran-dependent process by the α and β importin subunits, which constitute the high-affinity NLS receptor used by many proteins to affect their nuclear import (26). Whereas the abundance of nuclear IGFBP-3 is regulated by ubiquitin/proteasome-dependent degradation (15), the molecular mechanisms underlying its proteasome-mediated destabilization are not yet well understood. Proteasomes are abundantly found in both nuclei and the cytosol of eukaryotic cells but...
virtually absent from the lumen of membranous organelles such as the endoplasmic reticulum (27), and this raises the question how IGFBP-3 can be degraded in a proteasome-dependent fashion. A possible solution to this paradox comes from the observations that extracellular IGFBP-3 can be internalized by cells (28), and this is correlated with nuclear localization of IGFBP-3. Another possibility is that IGFBP-3 reaches the nucleus from its synthesis spots via an intracrine pathway as proposed for actions of non-secreted IGFBP-5 (29); however, there is currently no evidence for such a pathway for IGFBP-3. Our results indicate that endogenous E7 and IGFBP-3 co-localize near the nuclear envelope and/or in the nuclear periphery in CaSki cells. E7 binds to the NLS of IGFBP-3 \textit{in vitro} and induces ubiquitin/proteasome-dependent destruction of nuclear \( \Delta l\)-IGFBP-3 in C33A cells. Accordingly, E7 induces a dramatic reduction in the half-life of nuclear \( \Delta l\)-IGFBP-3 in C33A cells and this can be specifically inhibited by the proteasome inhibitor epoxomicin, whereas the metabolic half-life of the predominantly cytosolic E7-non-binding mutant \( \Delta l\)-IGFBP-3KGRKR228-232MDGEA is not altered in this assay. Our demonstration that E7 significantly enhances polyubiquitination of nuclear \( \Delta l\)-IGFBP-3, whereas polyubiquitination of the E7-non-binding mutant \( \Delta l\)-IGFBP-3KGRKR228-232MDGEA is not altered by E7, establishes nuclear \( \Delta l\)-IGFBP-3 as a target for E7-induced ubiquitin/proteasome-dependent destruction. Consistent with the low endogenous nuclear IGFBP-3 levels in the HPV-16-positive CaSki cells, E7 is expressed in the nucleus of these cells. Both endogenous proteins E7 as well as IGFBP-3 co-localize in nuclear fractions derived from CaSki cells and the nuclear fraction of endogenous IGFBP-3 accumulates with epoxomicin treatment (Figure 3A). Moreover, IGFBP-3 can be stabilized by epoxomicin in areas around the nuclear envelope/periphery of CaSki cells (Figure 2C). These findings suggest that endogenous E7 can induce proteasome-dependent destruction of endogenous nuclear IGFBP-3 in the nuclear periphery of CaSki cells. In keeping with these results, endogenous nuclear IGFBP-3 is only detectable in very low levels in the vast majority of tumour cells analysed so far (4). It should be noted that the present study rules out that E7 may also enhance degradation of cytoplasmic IGFBP-3. It is

![Figure 4. HPV-16 E7 reduces the half-life of nuclear IGFBP-3.](https://academic.oup.com/carcin/article-abstract/28/12/2511/2476415)
possible that at least a part of nuclear IGFBP-3 is reduced by its enhanced degradation in the cytoplasm by E7, as the endogenous IGFBP-3 levels are also increased in the membrane fraction of epoxomicin-treated CaSki cells (Figure 3A). Moreover, since E7 can not bind to the predominantly cytoplasmic mutant Dls-IGFBP-3KGRKR228-232MDGEA, more work is necessary to address the question whether E7 can also induce the degradation of cytoplasmic IGFBP-3.

A role of the proteasome in E7-induced degradation of the retinoblastoma protein (pRb), another important nuclear E7 target protein (3), is well established. E7 targets pRb to proteasome-dependent degradation, which impairs pRb-dependent growth arrest in the G1 phase of the cell cycle (30). Moreover, it was shown that the abundance of pRb is inversely correlated with E7 oncoprotein expression in high-grade cervical lesions, whereas pRb nearly completely disappeared in invasive cervical carcinomas in situ (2), underlining the importance of E7-mediated degradation of pRb in cervical carcinogenesis. Although the underlying mechanism is still little understood, it has been shown that E7 can interact with the S4 adenosine triphosphatase subunit of the 19S regulatory complex of the 26S proteasome which may circumvent the need for polyubiquitination (31). More recently, it was shown that E7 can also induce polyubiquitination and proteolysis of pRb through the ubiquitin/proteasome pathway (32). These findings suggest that E7-induced proteasome-dependent proteolysis of pRb probably involves more than one pathway, and the role of these pathways for the degradation of IGFBP-3 warrants further study.

Abrogation of IGFBP-3 induced apoptosis by HPV-16 E7

Expression of the IGFBP-3 gene is induced by the tumour suppressor proteins p53 (5) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (33), as well as by several pro-apoptotic and growth inhibitory factors, like transforming growth factor-β (6) and retinoic acid (34), suggesting that IGFBP-3 itself might act as a tumour suppressor, presumably via its ability to halt proliferation and induce apoptosis in cancer cells. IGFBP-3 can induce apoptotic cell death by several pathways (reviewed in refs 4 and 7). It can modulate the anti-apoptotic effects of IGF-I by regulating the IGF-I–IGF-R-I interaction (reviewed in refs 4 and 7). Moreover, induction of programmed cell death through IGF-R-I-independent mechanism (6,8,9,35,36) and by exclusively intracellular cytoplasmic (15,37) as well as nuclear IGFBP-3 (14,15) has been demonstrated (reviewed...
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in refs 4 and 7). The results of this study, that secreted IGFBP-3 wild-type and nuclear as well as cytotoxic IGFBP-3 mutants can induce apoptotic cell death in C33A cervical cancer cells, are in keeping with these earlier findings. Co-expression of E7 leads to abrogation of apoptosis induced by nuclear IGFBP-3; however, apoptotic cell death induced by a predominant cytotoxic E7-non-binding mutant of IGFBP-3 is not blocked by E7 in C33A cells. These results suggest that the ability of E7 to block IGFBP-3-induced apoptosis in cervical cancer cells involves direct binding and inactivation of IGFBP-3 by E7. Consistent with the previous studies, showing that the IGFBP-3 protein levels and metabolic stability are reduced in E7-immortalized keratinocytes (16) and in HPV-16-positive tumorigenic cell lines (38), the present data suggest that immortalization of human cells by HPV-16 may involve the inhibition of the pro-apoptotic activity of nuclear IGFBP-3. This finding is in keeping with the earlier studies demonstrating that the expression of E7 suppresses apoptosis in several cell types (39), including human prostate cancer cells (16), normal human fibroblasts (40) and human keratinocytes (41). However, many other studies showed that E7 can also induce a strong apoptotic response in several experimental systems (39), indicating that the regulation of apoptosis by E7 is complex and may involve more than one regulatory pathway. Together, the results reported in the present study suggest that destruction and thereby functional inactivation of nuclear IGFBP-3 contribute to the suppression of apoptosis by E7.

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