

The human papillomavirus (HPV) E6 oncoproteins promotes nuclear localization of active caspase 8

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ABSTRACT

The HPV-16 E6 and E6* proteins have been shown previously to be capable of regulating caspase 8 activity. We now show that the capacity of E6 to interact with caspase 8 is common to diverse HPV types, being also seen with HPV-11 E6, HPV-18 E6 and HPV-18 E6*. Unlike most E6-interacting partners, caspase 8 does not appear to be a major proteasomal target of E6, but instead E6 appears able to stimulate caspase 8 activation, without affecting the overall apoptotic activity. This would appear to be mediated in part by the ability of the HPV E6 oncoproteins to recruit active caspase 8 to the nucleus.

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Introduction

Human papillomaviruses (HPVs) are the causative agents of cervical cancer (Bouvard et al., 2009). The virus encodes two oncoproteins, E6 and E7, which directly contribute towards the initiation of tumour formation and the maintenance of tumour development (Androphy et al., 1987; Banks et al., 1987; Smotkin and Wettstein, 1986). Loss of expression or inhibition of E6/E7 function in cells derived from cervical tumours results in a cessation of transformed cell growth and induction of either senescence or apoptosis (Butz et al., 1996, 2003; Jabbar et al., 2012; von Knebel Doeberitz et al., 1992). Therefore both E6 and E7 represent excellent targets for therapeutic intervention in HPV-associated malignancy. Both E6 and E7 exert their transforming activities through multiple mechanisms. In the case of E7, this involves inactivation of essential regulators of cell cycle control, of which the pRb family of pocket proteins are the most well-defined (McLaughlin-Drubin and Munger, 2009). The E6-induced inactivation of p53 and of PDZ domain-containing targets is also believed to play critical roles in the ability of the virus to induce transformed phenotypes (Banks et al., 2012; Moody and Laimins, 2010). However additional mechanisms are also likely to be involved, as evidenced by the broad array of novel interacting partners recently demonstrated in a variety of proteomic analyses of both the E6 and

E7 oncoproteins from multiple HPV types (Rozenblatt-Rosen et al., 2012; White et al., 2012).

Although there are close to 200 different HPV types (zur Hausen, 2009), only a small subset of these, the so-called high-risk types, cause cervical cancer (Muñoz et al., 2003). A great deal of effort has been expended in trying to identify the unique characteristics of these high-risk viruses that might be responsible for tumour development. One such potentially relevant feature of this group of viruses is the alternative splicing of the E6 ORF, which generates multiple short forms of the E6 proteins, termed E6* (Smotkin et al., 1989). In the case of HPV-18 E6*, only one such isoform is produced, whilst in the case of HPV-16 E6* at least three such isoforms have been reported (Doorbar et al., 1990; Roggenbuck et al., 1991). The functions of E6* are still a matter of intense debate. In some cases they appear to be able to negatively regulate the activity of the full-length E6 proteins (Pim and Banks, 1999; Pim et al., 1997), whilst in others they seem to have intrinsic functions of their own, and potentially modulate a variety of different signal transduction pathways (Pim et al., 2009). A series of recent studies provided indications that the HPV-16 E6 and E6* proteins can differentially affect caspase activity, an effect related to their ability to interact with caspase 8 and, in the case of full length E6, target it for degradation (Filippova et al., 2007; Tungteakkun et al., 2010). These studies focused on the effects of E6 and E6* on cell survival and apoptosis induction pathways; however recent studies have also shown that caspase activation is an important aspect of the normal viral life cycle, appearing to be essential for the later stages of the viral life cycle in differentiating keratinocytes (Moody et al., 2007). We have

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therefore been interested in investigating how conserved was the ability of different HPV E6 proteins, and their alternatively spliced isoforms, to interact with and modulate caspase 8 activity.

Results

E6 from different HPV interact with pro-caspase 8 and DED in vitro

Previous studies had shown that HPV-16 E6*, and to a much lesser extent the full length HPV-16 E6, could interact with caspase 8 through the death effector domain (DED) (Tungteakkhun et al., 2010). Since modulation of caspase activation appears important for the viral life cycle (Moody et al., 2007), we were interested in determining whether the E6 proteins from other HPV types could also associate with caspase 8 in a similar manner. To do this, we performed a series of GST pull-down assays using purified GST fusion proteins of HPV-11 E6, HPV-16 E6, HPV-18 E6 and the two corresponding E6* proteins. Full-length caspase 8 was in vitro translated and radiolabelled with ^{35}S -methionine, and then incubated with the GST fusion proteins. Interaction assays were done at 4 °C, to reflect the conditions used in previous studies (Filippova et al., 2007), and also at room temperature. Following extensive washing the bound proteins were analyzed by SDS PAGE and

autoradiography. The results in Fig. 1A demonstrate that all the E6 proteins analyzed showed a similar capacity to interact with the caspase 8, although HPV-18 E6* appeared to interact somewhat more strongly. Surprisingly, and in contrast to previous studies, we also observed association between HPV-11 E6 and caspase 8, although in all cases interaction was significantly stronger at room temperature than at 4 °C, demonstrating that the ability of E6 to bind to caspase 8 is cold sensitive. To extend these analyses to determine whether the site of interaction was mediated via the DED, we repeated the assays using the isolated in vitro translated radiolabeled caspase 8 DED. The results in Fig. 1B again demonstrate a similar level of interaction between the different E6 proteins and the DED, which is, again, cold sensitive. Again, high levels of interaction are seen with both the full length and E6* proteins, as well as with HPV-11 E6.

HPV-18 E6* interaction with DED is carboxy terminal domain dependent

Previous studies had shown a significant difference in the ability of HPV-16 E6* and HPV-16 E6 to interact with caspase 8 (Filippova et al., 2007). In our analysis we noticed a slight increase in the capacity of HPV-18 E6* to interact with caspase 8 compared with the full length E6 protein. To investigate the mechanism by

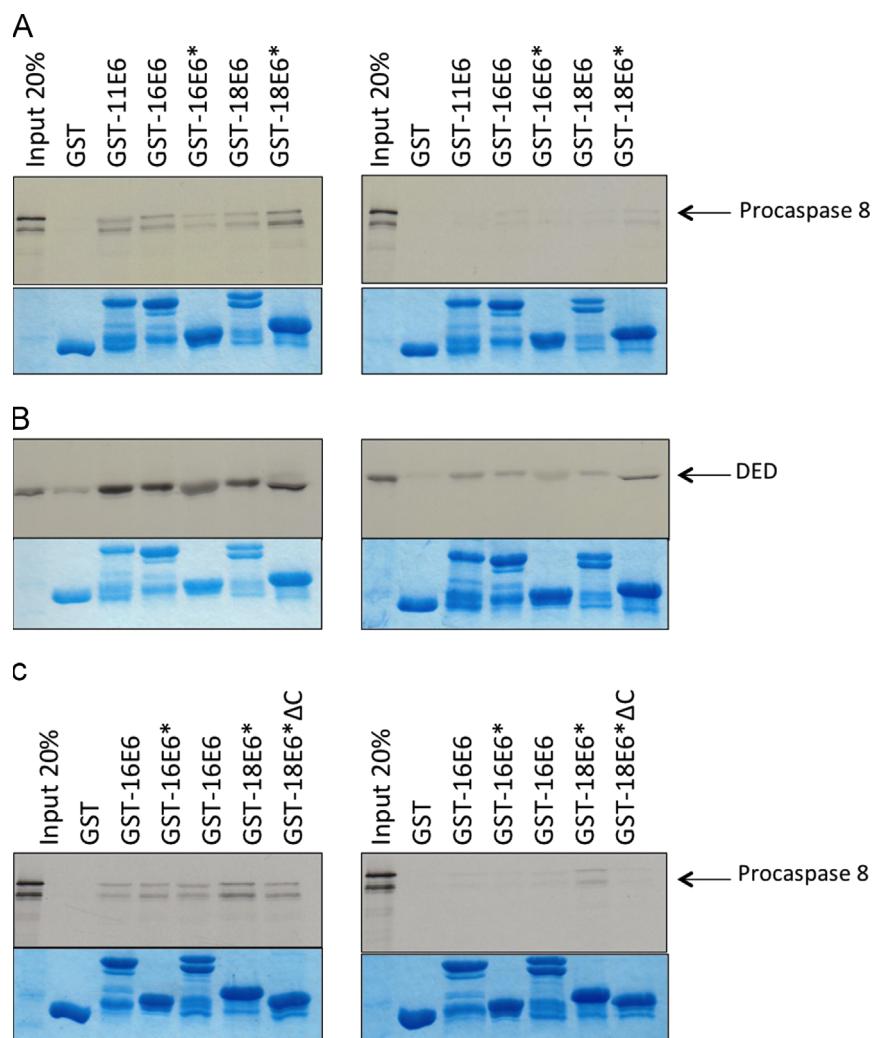


Fig. 1. Different HPV E6 proteins interact with the caspase 8 DED. The different HPV E6 fusion proteins were purified and incubated with *in vitro* translated and radiolabelled full-length caspase 8 (Panels A and C) or the isolated DED (Panel B) at either room temperature (left hand panels) or at 4 °C (right hand panels). After extensive washing the bound proteins were analyzed by SDS PAGE and autoradiography. Arrows highlight the bound caspase 8 or DED whilst the lower section in each Panel shows the Coomassie stained gel to show similar levels of GST fusion protein expression. The 20% inputs of the caspase 8 and DED are also shown.

which this could occur, we repeated the assay using wild type E6* plus a mutant where the carboxy terminal 8 unique amino acids were deleted. As can be seen from Fig. 1C, HPV-18 E6* does interact slightly more efficiently with caspase 8 compared with the full length E6 protein at both room temperature and 4 °C. Furthermore, this increased association would appear to require the amino acid residues lying within the unique carboxy terminal stretch of E6*. These results demonstrate that interaction between E6 and caspase 8 is conserved across both low- and high-risk HPV types, and, in the case of HPV-18 E6*, involves sequences that are unique to the carboxy terminal region of the protein.

E6 induces caspase 8 activation

In an extensive series of in vitro degradation assays, none of the above E6 proteins were found to have any effect upon the stability of caspase 8 (data not shown). Therefore we proceeded to investigate the effects of E6 upon the levels of caspase 8 expression in vivo. HEK293 cells were transfected with a caspase 8 expression plasmid, together with either HA-tagged HPV-18, HPV-16 or HPV-11 E6 expression plasmids. After 24 h the cells were harvested and the pattern of caspase 8 activation monitored by Western blotting using anti-caspase 8 antibody. As can be seen from Fig. 2A, the different E6 proteins slightly reduced the total levels of full-length (p57) caspase 8 expression. However, the amount of cleaved active (p18) caspase 8 increased significantly, in the presence of the full length

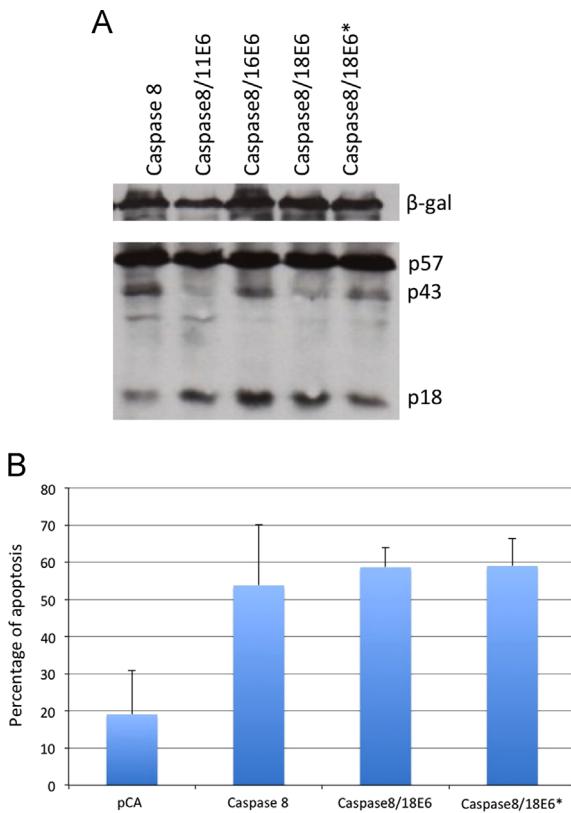


Fig. 2. HPV E6 proteins promote activation of caspase 8. Panel A. HEK293 cells were transfected with a caspase 8 expression plasmid either alone or together with the different E6 expression plasmids as indicated. After 24 h the cells were harvested and the levels of caspase 8 expression ascertained by Western blot analysis. Indicated is the full-length form of caspase 8 (p44) and the active cleaved form (p18). Also shown is the transfection and loading efficiency control of β -gal. Panel B. HEK293 cells were transfected with caspase 8 either alone or with HPV-18 E6 expression plasmids, as indicated, and after 24 h the cells were harvested and levels of apoptosis ascertained by Annexin V staining and FACS analysis. The results show percentage apoptosis from three separate experiments together with the standard deviations.

HPV-16 and HPV-18 E6 proteins, as well as with HPV-11 E6. In addition, a modest increase in caspase 8 activation was also observed with HPV-18 E6*. These results indicate that, although E6 does not significantly affect total caspase 8 levels in transient transfection experiments, it can nonetheless affect the degree to which caspase 8 is activated.

E6 binding to pro-caspase 8 does not induce apoptosis

Caspase 8 is the initiator caspase in the induction of the apoptotic cascade (McIlwain et al., 2013). Therefore we performed a series of assays to investigate whether the levels of increased caspase 8 activation seen in the presence of E6 was sufficient to increase the levels of apoptosis induced by caspase 8 overexpression. Cells were transfected with caspase 8, in the presence and absence of HPV-18 E6 and HPV-18 E6* expression plasmids, and after 24 h the levels of apoptosis were assessed by Annexin V binding and FACS analysis. As can be seen from Fig. 2B, E6 expression had a minimal effect on the levels of apoptosis induced by caspase 8, indicating that the increase in caspase 8 activation induced by E6 in Fig. 2A does not result in increased levels of apoptosis.

E6 induces caspase 8 nuclear translocation

Previous studies have shown that caspase activation is an important step in the HPV life cycle (Moody et al., 2007; Terenzi et al., 2008; Yu et al., 2007). Caspases are found primarily in the cytoplasm (Zhivotovsky et al., 1999) whilst E6 oncoproteins are expressed throughout the cell. We were therefore interested in determining whether E6 could in any way modulate the localization of caspase 8 within the cell. To do this, U2OS cells were transfected with a caspase 8 expression plasmid in the presence and absence of the HA-tagged HPV E6 expression plasmids. After 24 h, the cells were fixed and analyzed for the presence of HA-tagged E6 proteins and the co-transfected caspase 8 by immunofluorescence and microscopy. The results in Fig. 3 demonstrate that E6 has a diffused distribution, with both nuclear and cytoplasmic patterns of expression, similar to that reported previously (Guccione et al., 2004; Vaeteewoottacharn et al., 2005). Caspase 8, when transfected alone, is also predominantly found within the cytoplasm (Fig. 3A and Supplementary Fig. 1A). In contrast, when caspase 8 is co-expressed with either HPV-18 E6, HPV-16 E6 or HPV-11 E6 there is a significant degree of co-localization (Fig. 3B and Supplementary Fig. 1B and 1C) between E6 and caspase 8. In addition in cells where E6 nuclear localization is apparent, there is a corresponding increase in the levels of nuclear caspase 8 expression. Similar results are also obtained with HPV-18 E6*, where, again, there is a significant degree of co-localization with caspase 8, and a significant re-localization of caspase 8 to the nucleus. These results demonstrate that caspase 8 activation induced by HPV E6 is accompanied by an increase in the amount of caspase 8 found within the nucleus.

The presence of caspase 8 in the nucleus of HeLa cells is E6 dependent

In order to determine whether the pattern of caspase 8 was similarly affected by endogenous HPV-18 E6, HeLa cells were transfected with siRNA against luciferase as a control or against HPV-18E6/E7. After 72 h the cells were extracted into cytosolic, membrane, nuclear and cytoskeletal pools, and the pattern of caspase 8 expression ascertained by Western blotting. The results obtained are shown in Fig. 4A and demonstrate strong accumulation of p53 in the nucleus of cells transfected with siE6/E7. In the case of active caspase 8, there is a clear signal present within the

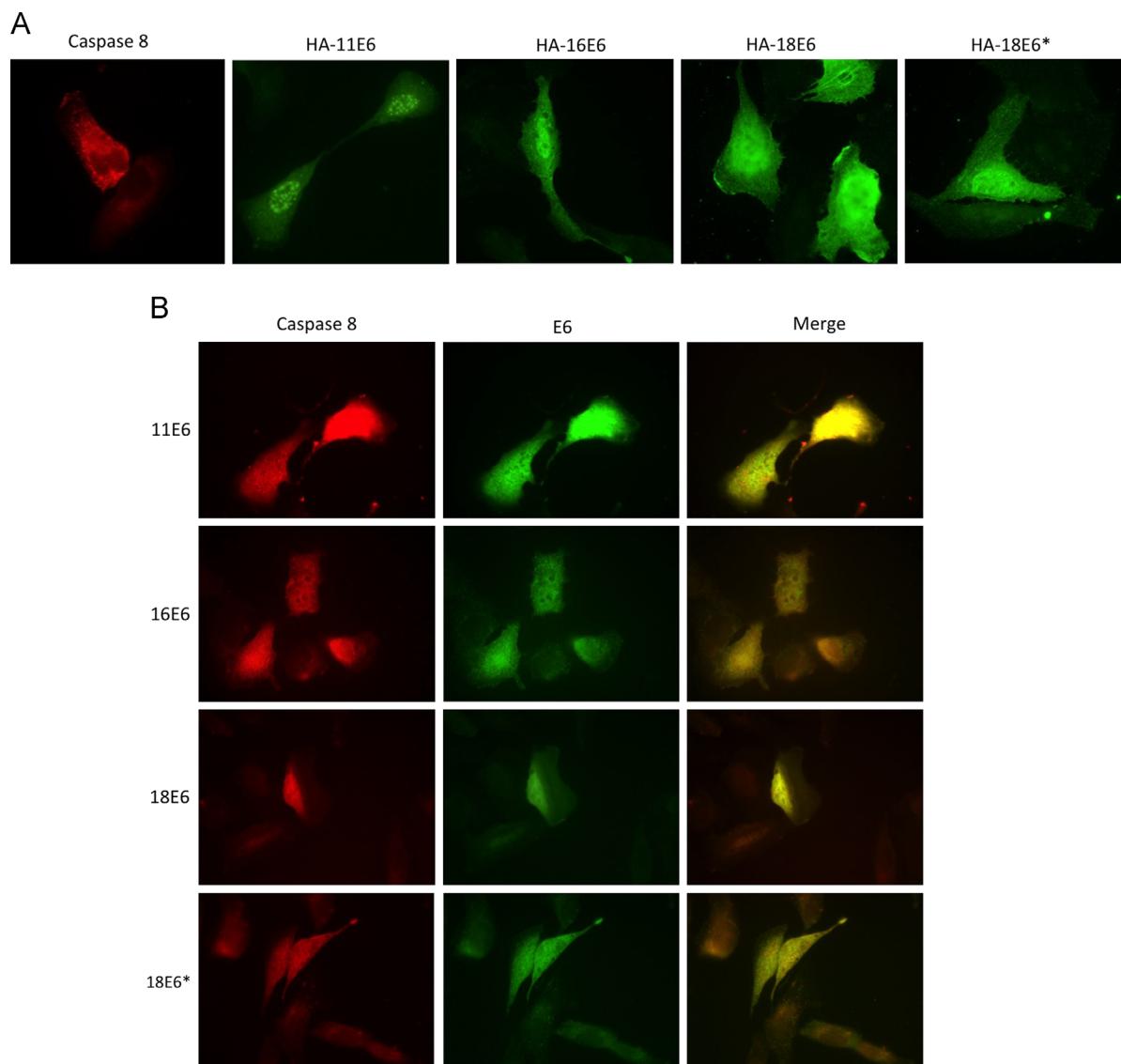


Fig. 3. HPV E6 modifies the subcellular distribution of caspase 8. U2OS cells were transfected with caspase 8 or the different E6 expression plasmids, either alone (Panel A) or in combination (Panel B) as indicated. After 24 h the cells were fixed and the patterns of caspase 8 expression ascertained by immunofluorescence analysis using anti-caspase 8 antibody and rhodamine-conjugated anti-mouse antibody (red), and HPV E6 detected by immunofluorescence analysis using anti-HA antibody and anti-rabbit Alexa 414 (green). Note the largely cytoplasmic distribution of caspase 8 and the nuclear/cytoplasmic distribution of the E6 proteins. Upon co-expression, there is marked co-localization of both proteins throughout the cell but an increase of caspase 8 in cells where E6 also displays nuclear accumulation.

nuclear fraction, and this decreases significantly upon siE6/E7 transfection.

We also performed immunofluorescence analysis of the patterns of caspase 8 expression in HeLa cells following siRNA ablation of E6/E7 expression. As can be seen from Fig. 4B, caspase 8 displays a predominantly cytoplasmic distribution in HPV negative HaCaT cells, whilst in control siRNA transfected HeLa cells caspase 8 has a significant proportion of nuclear localization. However, following transfection with siRNA E6/E7 there is a significant accumulation of cytoplasmic caspase 8. These results demonstrate that there are increased levels of nuclear caspase 8 in cells expressing HPV E6 oncoproteins.

Discussion

The ability of the high-risk HPV E6 oncoproteins to contribute towards the development of cervical cancer requires multiple

functions, a number of which are likely to be absent from the low risk HPV E6 oncoproteins (Rozenblatt-Rosen et al., 2012; White et al., 2012). The capacity of high-risk HPV E6 oncoproteins to exist as an alternatively spliced shorter isoform termed E6* is one such unique feature of the high-risk HPV types (Doorbar et al., 1990; Smotkin et al., 1989). Although the E6* proteins do not appear to play a major role in the capacity of these HPV types to bring about cell immortalization or transformation (Sedman et al., 1991), they do nonetheless have a number of other intriguing activities. One of these is their ability, in conjunction with the full-length E6 proteins, to interact with and potentially modulate, the activity of caspase 8 (Filippova et al., 2007; Tungteakkun et al., 2010). In the case of HPV-16, E6* appeared to enhance caspase 8-induced apoptosis, whilst the full-length E6 protein appeared capable of directing caspase 8 degradation. Thus, changes in the ratios of expression of the two forms of E6 could be expected to play a major role in viral pathogenesis. In the present study, we have found that interaction with caspase 8 is common to E6

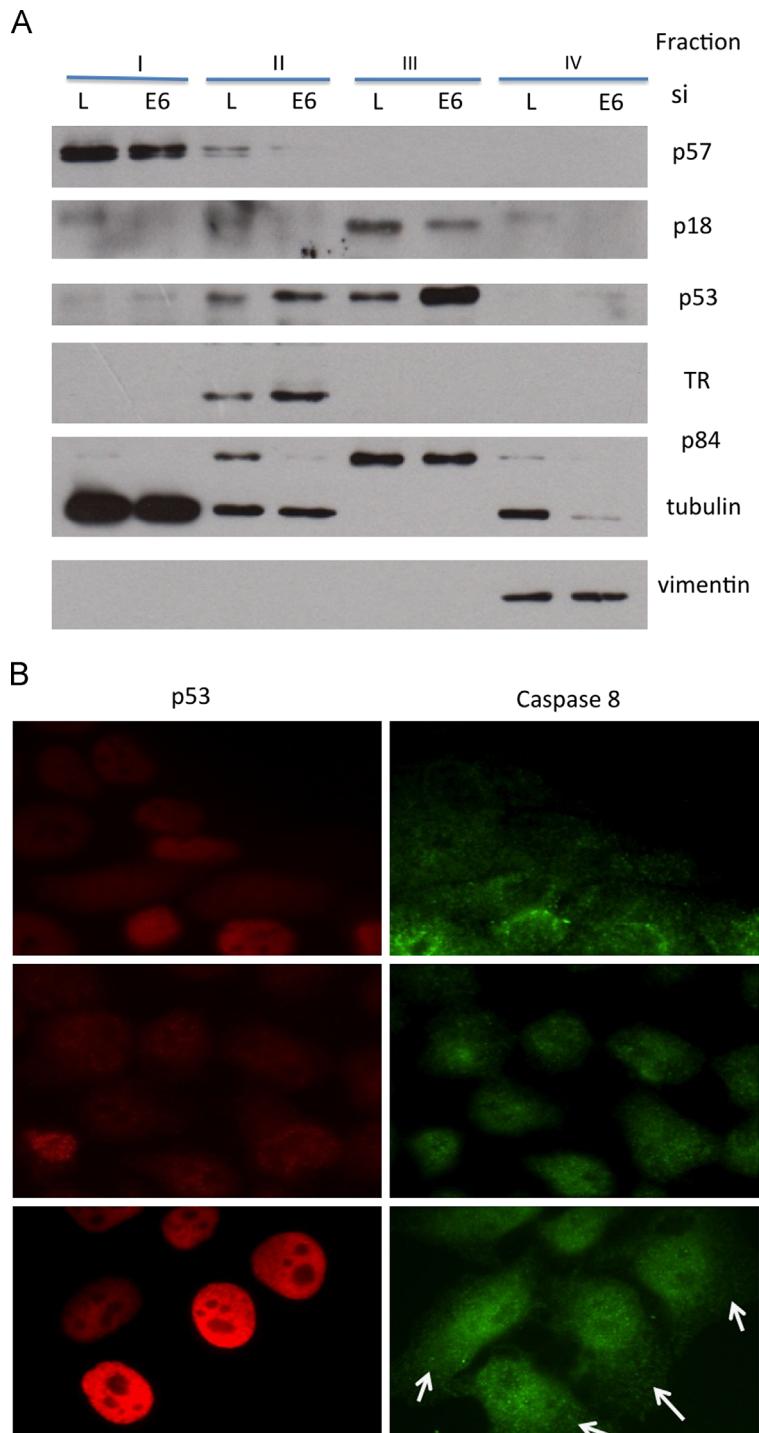


Fig. 4. HPV-18 E6 induces active caspase 8 expression in the nucleus of HeLa cells. Panel A. Cells were transfected with siRNA luciferase (L) or siRNA E6/E7 (E6) and after 72 h the cells were harvested and fractionated into cytoplasmic (I), membrane (II), nuclear (III) and cytoskeletal pools (IV). The fractions were then subjected to Western blot analysis for caspase 8 (p57 full length and active p18) and p53. Markers for loading and fraction integrity were vimentin, tubulin, p84 and transferrin receptor (TR). Panel B. HeLa cells were transfected with siRNA luciferase (si luc) or siRNA E6/E7 (si E6) and after 72 h cells were fixed and stained for p53 and caspase 8. HaCaT cells were included for comparison. Note the strong increase in nuclear p53 following E6/E7 ablation and the corresponding increase in cytoplasmic caspase 8, which is indicated by the arrows.

proteins of both high and low risk types, and that one consequence of this association is the stimulation of caspase 8 activity and concomitant re-localization to the nucleus.

The ability of different HPV E6 oncoproteins to interact with caspase 8 appears to be quite highly conserved. Using simple GST pull-down assays we found that high risk HPV-16 and HPV-18 E6 proteins, as well as low risk HPV-11 E6, could all interact with caspase 8. This interaction also seemed to involve a similar mode

of recognition, with interaction appearing to be mediated via the caspase 8 DED. Interestingly, E6 proteins appear to interact more strongly with the isolated DED than with the full-length caspase 8. The reasons for this are unclear but could involve conformational changes in the DED once it is removed from the activated caspase, or be indirect and be a reflection of the tendency for an isolated DED to aggregate (Mielgo et al., 2009), and thereby increase the amount complexed with E6. Further studies will be required to

investigate these possibilities. Furthermore, in all cases the interaction between E6 and caspase 8 was cold sensitive, with only minimal levels of association detectable at 4 °C. Interestingly, we did not detect much difference between the abilities of HPV-16 E6 and HPV-16 E6* to interact with caspase 8, although HPV-18 E6* showed a modest but significantly increased potential to associate with caspase 8 compared with full length HPV-18 E6. Interestingly, this increased capacity to bind to caspase 8 appeared to be mediated through the unique carboxy terminal 8 amino acids of HPV-18 E6* protein.

A large number of HPV E6 substrates are directed for degradation at the 26S proteasome (Scheffner et al., 1993; for review see Banks et al., 2003), although in the case of caspase 8, E6 has little effect on the total levels of caspase 8 expression in cell. However the E6 proteins, and to a lesser extent E6*, all seem capable of stimulating caspase 8 cleavage to the active form of the enzyme. This would initially seem counterintuitive, as it might suggest that E6 was capable of stimulating caspase 8-driven apoptosis. However this does not appear to be the case, and this is most likely due in part to the ability of E6 to alter the subcellular distribution of caspase 8. Thus, caspase 8 when expressed alone is found primarily within the cytoplasm, whilst in the presence of E6 and E6* there is a marked increase in the amount of caspase 8 found in the nucleus. This occurs when E6 proteins are overexpressed and co-transfected with caspase 8, but is also observed with endogenous caspase 8 in HeLa cells, where there are high levels of nuclear caspase 8 which becomes much more cytoplasmic following siRNA ablation of E6/E7 expression. This nuclear accumulation of caspase 8 is in agreement with previous studies that reported increased levels of caspase 8 in the nucleus of HPV positive tumour cell lines and cervical cancers (Arechaga-Ocampo et al., 2008). Our results now indicate that this is most likely due to the activity of the E6 oncoprotein. These results suggest that E6 most likely recruits caspase 8 to the nucleus to perform functions that are beneficial either for the viral life cycle, or in the maintenance of cell proliferation in E6 transformed cells. What these activities are remains unclear, but could involve modulation of apoptotic pathways depending upon the levels of E6 expression (Filippova et al., 2007), or the targeting of substrates that would not normally be recognized by the caspase in these particular cellular locations. One such candidate would be the viral E1 protein, which is cleaved by caspases as part of the normal viral life cycle (Moody et al., 2007) and it will now be of interest to determine whether E6 has any potential role in this activity.

Materials and methods

Cells and transfections

Hek-293, HeLa, U2-OS and HaCaT cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were done by the calcium phosphate method or by FuGENE® HD Transfection Reagent (Promega) according to the manufacturer's instructions.

Plasmids

HA-tagged HPV E6 expression plasmids were described previously (Thomas et al., 2013). Pro-caspase 8 expression plasmid was obtained by cloning the pro-caspase 8 sequence into the pcDNA3 vector. The pCA-DED construct was obtained by cloning the coding sequence of Pro-caspase 8 death effector domain into the pCA backbone using standard PCR (BamHI and EcoRI sites). The products were verified by sequencing.

GST-fusion proteins purification

GST-fusion proteins production and purification were described previously (Pim et al., 1997).

In vitro translation and in vitro binding assays

An amount of 1 µg pro-caspase 8 or DED expressing plasmid was in vitro transcribed and translated using the TNT T7 Coupled Reticulocyte Lysate System® (Promega) employing [³⁵S]-methionine according to the manufacturer's instructions. For the *in vitro* binding assays equal amounts of the different E6 GST-fusion proteins were incubated with either *in vitro* translated pro-caspase 8 or *in vitro* translated DED in PBS at 4 degrees or room temperature for 90 min. After extensive washing with 0.2% NP-40/PBS the bound proteins were analyzed by SDS-PAGE and autoradiography. GST-fusion proteins amount was analyzed by Coomassie staining.

Immunofluorescence and cell imaging

About 24 h after transfection U2-OS cells were fixed in 3% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton-X100, the cells were then incubated for 2 h at 37 °C with polyclonal anti-HA probe (Santa Cruz biotechnology), monoclonal anti caspase 8 (IC12, Cell Signalling) either alone or in combination, washed extensively with PBS, and incubated with anti-mouse or anti-rabbit conjugated to fluorescein or rhodamine (Molecular Probes) at 37 °C. Slides were washed and mounted in Vectashield mounting medium (Vector Laboratories). HeLa and HaCaT cells were processed in the same manner 72 h post siRNA transfection and were incubated with anti p53 (Santa Cruz) and anti-caspase 8 antibody (IC12, Cell Signalling). Slides were analyzed with either a Leica DMLB fluorescence microscope with a Leica photo camera (A01M871016) or a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60 × objective oil immersion lens.

siRNA silencing and fractionation experiments

HeLa cells were seeded in 6 cm diameter dishes and maintained for 24 h to allow attachment, then cells were transfected with siRNA (Dharmacon) against E6/E7 (5'CAUUUACCG CCCGACGAG) and Luciferase as a control, using the Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. About 72 h after silencing, cells were harvested and protein extracted using the sub-cellular proteome extraction kit (Calbiochem) according to the manufacturer's instructions, protein levels were evaluated by Western blot.

Western blotting and antibodies

After 24 h post transfection, cells were washed and directly lysed in sample loading buffer to generate a total cell extract which was analyzed by SDS-PAGE and Western blotting onto a 0.22 µm nitrocellulose membrane (Schleicher and Schuell). Membranes were blocked at 37 °C in 7% milk/0.1% Tween 20/PBS for 1 h, followed by incubation with the appropriate primary antibody diluted in 5% milk/0.1% Tween 20/PBS. After washing three times with 0.1% Tween 20/PBS membranes were incubated with HRP-conjugated anti mouse (DAKO) in 5% milk/0.1% Tween 20/PBS for 1 h. Blots were developed using Amersham ECL reagents according to manufacturer's instructions. Primary antibodies were used as follows: anti-Caspase 8 (IC12) (Cell signalling) at 1:1000 which recognizes both the active and inactive forms of the

enzyme, anti-HA (Roche) at 1/1000 and anti- β -galactosidase (Promega) at 1/5000, anti p53 (Santa Cruz) at 1:1000.

Annexin V assay

HEK293 cells were seeded in a 6 cm diameter dish and transfected with a plasmid expressing Procaspsase 8, either alone or in combination with HPV-18 E6 and E6* plasmids. After 24 h the cells were washed and trypsinized gently. They were then washed once in DMEM/10%FBS and once in PBS. The amount of apoptosis was then determined by incubating the cells with Annexin V-FITC using the Annexin-V-FLUOS Staining kit (Roche) according to the manufacturer's instructions. The cells were then analysed using a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2013.12.013>.

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