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Human papillomavirus type 16 E5 protein colocalizes with the antiapoptotic Bcl-2 protein

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Summary. Human papillomavirus type 16 E5 protein contributes to cellular transformation by increasing the mitogenic stimulus from growth factor receptors to the nucleus. In order to study the biological mechanisms of the E5 protein we performed site-directed mutagenesis of the E5 gene. Wild-type as well as mutant E5 proteins were transiently expressed in human cervical epithelial cells, and cell morphology, expression of proteins involved in cell adhesion, and localization of the different proteins were studied. Little differences in cell morphology or expression kinetics were observed between the different E5 proteins, except for relocalization of a mutant E5 protein where a hydrophobic leucine membrane anchor was mutated to positively charged amino acids. This mutant E5 protein localized to lamellipodia, which are motility-associated structures at the leading edge of motile cells. In our experimental conditions, 100% of E5-expressing epithelial cells died by four days of expression, possibly due to toxicity or disturbance of the membrane compartment by the E5 protein. Most interestingly, a remarkable colocalization of the E5 protein with the Bcl-2 antiapoptotic protein on intracellular membranes was established.

Introduction

The E5 open reading frame (ORF) of human papillomavirus type 16 encodes a 83 amino acid long transmembrane protein [4, 22]. Expression of the E5 protein leads to enhanced signaling from the epidermal growth factor (EGF) receptor in response to EGF ligand binding by direct activation of signal transduction pathways and by enhanced recycling of the receptor to the plasma membrane [10, 11, 12]. Direct binding of the HPV 16 E5 protein to the EGF receptor (EGFR)

has been suggested on the basis of overexpression experiments in COS cells [25]. Signaling is mediated through several pathways, e.g. the MAP kinase and the protein kinase C pathways [11, 12, 21]. The E5 protein binds to the 16 kDa subunit of vacuolar H^+-ATPase and inhibits the acidification of endosomes, which is essential for the degradation of endocytosed receptor-ligand complexes [10, 43]. Binding to 16 kDa subunit contributes to E5 function but was recently shown not to be sufficient for the disruption of 16 kDa proton pump function [1, 2]. Further, it has been shown that EGFR activation by E5 can be functionally dissociated from 16 kDa binding [38].

HPV 16 E5 protein localizes in the Golgi apparatus, ER, nuclear membrane, and in cytoplasmic vesicles [10, 35]. Golgi localization of bovine papillomavirus (BPV) type 1 E5 protein is essential for platelet-derived growth factor receptor (PDGFR) – dependent transformation of cells [7, 41], indicating a close relationship between localization and function. BPV E5 binds PDGFR through a glutamine residue, which is critical for the oncogenic function of BPV E5 but is not conserved in the HPV 16 E5 protein [19, 20, 30, 31]. Recent findings suggest an alternative mechanism of BPV E5 transformation through activation of the PI-3 kinase, which does not require Golgi localization of BPV E5 [44].

Inhibition of gap-junctional intercellular communication (GJIC) between epithelial cells may contribute to cellular transformation and is known to occur at the early stages of carcinogenesis [49]. Inhibition of GJIC has been reported in the case of HPV 16 and BPV 4 E5 proteins [16, 34]. However, in a recent report GJIC downregulation and cellular transformation by BPV 4 E5 were reported to be two independent functions [3], showing the complexity of E5 protein functions.

In the present study we constructed a series of HPV 16 E5 mutants by site-directed mutagenesis in order to study the biological functions of the protein. We show here that the functions of the E5 protein are not mediated by morphological alterations in the actin cytoskeleton, microtubulus network, or adhesion to the extracellular matrix. In our experimental setting, expression of the E5 protein for 96 hours leads to cell death, but no involvement of apoptosis could be established. Disruption of a hydrophobic leucine anchor sequence within the N-terminus revealed an important domain anchoring the E5 protein to its intracellular localization. Most importantly, the E5 protein was shown to colocalize with the Bcl-2 antiapoptotic protein in the intracellular membranes, particularly ER.

Materials and methods

Expression constructs

The HPV 16 E5 ORF was subcloned, as described in Oetke et al. [35], by PCR into the pEGFP-C1 vector (Clontech, Palo Alto, CA). The E5 ORF was also fused to the 10-amino-acid BPV 1 E2 hinge epitope in the 3F12-pCG vector (Quattromed, Tartu, Estonia) [28]. Site-directed mutagenesis was performed by PCR using two complementary oligonucleotides (Table 1; modified from [24]). The GFP as well as dsRed-N1 and dsRed-C1 vectors (Clontech) were used as control plasmids. The expression plasmids for wild-type, constitutively activated (Q61L), and dominant negative (T17N) forms of Rac1 provided with the myc-epitope were

Table 1. HPV 16 E5 mutants and their putative altered functions

Name	Change	Putative effect
M1	R58A C59A	abolish dimerization
M2	C14A C28A C30A	abolish dimerization
M3	C24A C26A	abolish dimerization
M5	Y39F	remove reactive OH-group
M6	Y63F Y68F	remove reactive OH-groups
M7	F57A F60A	neutralize phenylalanines to alanines
M9	S8A T9A T10A	disrupt phosphorylation
M10	L22K L23K	change membrane anchor to positive charge

kindly provided by Dr. A. Hall. The Bcl-2 and Bcl-x_L constructs were a gift from Dr. Xiaodong Li.

Cells and transfections

HeLa human epithelial cells were grown on coverslips in modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. Transient transfections were performed using FuGene (Roche Molecular Biochemicals, Mannheim, Germany), and the cells were fixed with 4% paraformaldehyde 22 h post transfection if not otherwise mentioned.

Immunofluorescent staining

Immunofluorescent staining was performed on fixed cells. Double antibody stainings were done sequentially, and phalloidin staining, where indicated, was performed after antibody incubations for 30′ at RT. The different E5 proteins as well as the GFP control were visualized with the help of the GFP epitope without an antibody staining, except for the E5 protein tagged with BPV E2 hinge. The antibodies were to LAMP-1, EEA-1, or Rab-7 (gifts), paxillin (Zymed, San Francisco, CA), vinculin (Serotec, Oxford, UK), FAK (Transduction Laboratories, Lexington, KY), Golgi 58 K protein (Sigma, Saint Louis, MO), β-COP (Sigma), GM130 Golgi protein (BD Pharmingen, San Diego, CA), Golgin-97 (Molecular Probes, Eugene, OR), β-tubulin (Sigma), myc-epitope (Upstate Biotechnology, Lake Placid, NY), BPV E2 hinge epitope (Quattromed), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-x_L (Santa Cruz), cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), and cytochrome c (Sigma). Filamentous actin was stained with rhodamin-labeled phalloidin

(Molecular Probes). ER and mitochondria were stained in living cells using the ER-Tracker and MitoTracker fluorescent probes (Molecular probes). Nuclei were stained with Hoechst 33342 (Molecular Probes).

Assays for apoptosis

To examine the possibility of apoptotic cell death, TMR red *in situ* cell death detection assay based on the TUNEL reaction (Roche Molecular Biochemicals) was performed followed by Hoechst nuclear staining. For positive control, apoptosis was induced by staurosporine treatment, and staining without enzyme was performed as a negative control for the TUNEL assay. A caspase inhibitor Z-VAD-FMK (Sigma) was used to study the putative apoptotic pathways involved. Cells were treated with 40 μmol/l inhibitor for 30′ and TUNEL staining was performed after 24, 48, 72, and 96 h. The capability of either Bcl-2 or Bcl-x_L antiapoptotic proteins to inhibit E5-induced cell death was studied in cotransfection experiments. Single transfections, GFP control transfections, as well as nontransfected cells were included in each experiment. Also, E5-transfected cells were stained for cytochrome c and the cleaved form of caspase-3. PARP cleavage was studied in immunoblotting using an antibody directed to PARP cleavage site (Biosource, Keystone, CO). DNA fragmentation in E5-transfected cells was studied by ethidium bromide staining of agarose gels. Coimmunoprecipitation experiments of E5 and Bcl-2 were performed with antibodies to Bcl-2 and GFP (Clontech).

Results

Expression of wild-type and mutant E5 proteins in human epithelial cells

HPV 16 E5 coding sequence was expressed as a GFP-E5 fusion protein [35]. There are no reliable antibodies available to the HPV 16 E5 protein and thus the GFP epitope was used to visualize the protein. Also, the E5 gene was cloned into the 3F12-pCG vector in order to utilize the BPV E2 hinge epitope, which is detectable with a monoclonal antibody [28]. This epitope was used to control for possible artefacts caused by the GFP epitope. The transfection efficiency for the GFP control plasmid was repeatedly shown to be higher than for the E5 expression plasmid. When transiently expressed in human epithelial HeLa cells, GFP was present throughout the cytoplasm and also in the nucleus, whereas GFP-E5 was at the ER, in small vesicles, and in the Golgi (22 h; Fig. 1B, 2C).

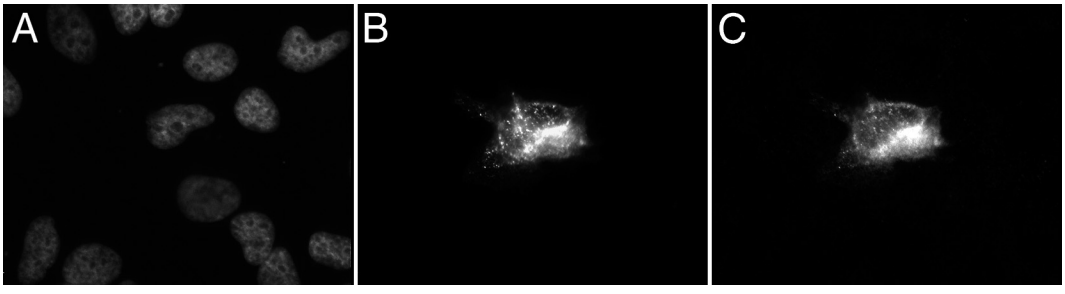

Fig. 1. Cotransfection of HeLa cells with expression constructs for GFP-E5 (**B**) and hinge-E5 (**C**) shows colocalization of both proteins. **A**, Hoechst nuclear stain

Cotransfection of GFP-E5 and hinge-E5 showed similar localization of the hinge-E5 protein and GFP-E5 confirming that the large GFP epitope did not affect E5 localization (Fig. 1). A considerable decrease in the number of positive cells and in the level of E5-expression took place with time, and by the end of the 96 h follow-up time no more E5-expressing cells could be found, suggesting lethal effects of E5 expression. This applied to both GFP- and hinge-tagged E5 proteins, whereas the expression of the GFP control plasmid remained similar throughout the 96 h follow-up. We also cotransfected cells with E5 together with one of the red fluorescent protein plasmids dsRed-N1 or dsRed-C1 to confirm the death of E5-expressing cells. The number of cells coexpressing E5 and red fluorescent protein decreased along with time, whereas the number of control cells coexpressing GFP and dsRed remained similar, confirming specific death of E5-expressing cells.

Point mutations within the E5 ORF were created by PCR. The spatial demands of each individual amino acid as well as the predicted secondary structure of the wild type protein were retained (Table 1). In M1, M2, and M3, cysteine residues capable of forming disulphur bridges were mutated to alanine. Dimerization has been established for BPV 1 E5 [40] and suggested for HPV 16 E5 [14, 29]. In M5 and M6 reactive hydroxyl groups of tyrosine residues putatively responsible for protein–protein interactions within the membrane compartment were changed to phenylalanine, which is spatially similar to tyrosine. A phenylalanine residue was mutated to neutral alanine in M7, and a putative serine-threonine–threonine casein kinase II phosphorylation site at residues 8–10 was replaced with alanines in M9. In M10 a hydrophobic leucine membrane anchor sequence was mutated to positively charged lysine residues ("positive-inside-rule"). Transfection efficiency, expression level, expression kinetics, and localization of wild-type and mutant E5 proteins were similar, except for M10. Therefore most figures represent the results with wild-type E5, M9, and M10.

Subcellular localization of the E5 proteins

Based on earlier observations made by us and by others we studied the putative Golgi localization of the different E5 proteins in HeLa cells. The E5 protein was visualized with the help of the GFP epitope without antibody staining. Partial colocalization of the E5 fusion proteins with the intermediate-Golgi 58 K protein was observed (GFP control, wtE5, M9, and M10 are shown in Fig. 2), whereas no colocalization was observed with GM130, or golgin-97. There were little differences in the subcellular localization between the different E5 proteins as the design of the mutations allowed us to expect (Table 1). In the case of M10, where a leucine membrane anchor was mutated to positively charged lysines, an additional peripheral localization to membrane lamellipodia, actin-rich structures at the leading edge of motile cells, was observed (Fig. 2G, arrows).

The partially vesicular fluorescence pattern of the E5 protein suggested the involvement of the endosomal machinery. However, staining of E5-expressing cells with antibodies to EEA-1, a marker for early endosomes [42], Rab-7 for late endosomes [9], or LAMP-1 for lysosomes [8], respectively, did not show

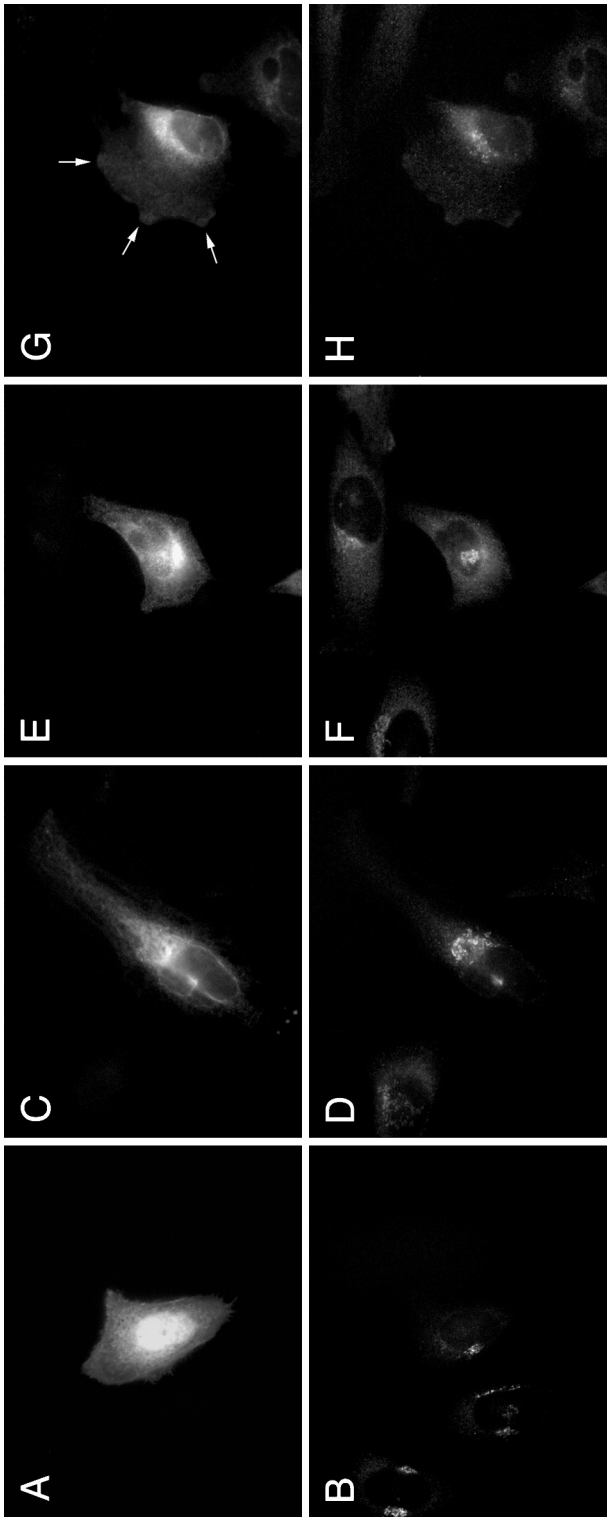

Fig. 2. E5 proteins show partial colocalization with the intermediate Golgi 58 K protein. (**A**, **C**, **E**, **G**), GFP fluorescence showing GFP alone (vector control), wild-type E5, M9, and M10, respectively; (**B**, **D**, **F**, **H**), staining for the intermediate Golgi 58 K protein. Arrows in (**G**) point to M10 localized in lamellipodia

colocalization with E5 fluorescence, although these structures were present in E5-expressing as well as in control cells. Nor was any colocalization seen with β-COP.

Effect of Rac1 GTPase on M10 localization

The small GTPase Rac1 plays a key role in the formation of lamellipodia [23]. In a cotransfection experiment using wtE5, M9, or M10 either alone or together with wild-type (wt), constitutively activated (Q61L), or dominant negative (T17N) Rac1, we aimed to study the mechanism responsible for the different intracellular localization of M10. When expressed alone, M10 was localized in lamellipodia, in the ER and in the Golgi (Fig. 2G). However, expression of M10 *per se* did not induce or enhance the formation of lamellipodia. Staining for F-actin was similar to nontransfected cells. Transfection with wtRac1 increased the number of lamellipodia, where M10 accumulated together with cotransfected Rac1 ((Fig. 3A, arrows; 3C). The cells contained stress fibers (Fig. 3B) depicting Rac1 phenotype. In cells expressing high levels of Q61LRac1, the M10 E5 protein was at the perinucleus (Golgi) and in vesicles, and also at the cell periphery (3D), while Q61LRac1 localized extensively to the plasma membrane (3F). Strong and dense F-actin stress fibers were observed (Fig. 3E). Peripheral localization of M10 in the cells expressing high levels of T17NRac1 was abolished (3G), and T17NRac1 showed mostly vesicular staining in the cytoplasm (3I). A reduction in actin stress fibers due to Rac1 inactivation was evident (Fig. 3H). Localization of wild-type E5 or mutant M9 was not affected by any of the Rac1 constructs indicating that lamellipodial localization and the effect of Rac1 activity on the localization of the protein is specific for M10.

Assays for cell death and colocalization of E5 with Bcl-2

Because we observed decreased amounts of E5-expressing cells along with time, we decided to study the involvement of apoptosis at different time points. Cells were TUNEL stained at 16, 18, 20, 22, 24, 48, 72, and 96 h post transfection. We observed less E5-expressing cells with time, and 96 h post transfection all E5-expressing cells had died. A chemical caspase inhibitor Z-VAD-FMK, or coexpression of either Bcl-2 or Bcl-x_L antiapoptotic proteins could not inhibit cell death. Nor did immunofluorescence for cytochrome c or cleaved caspase-3, or immunoblotting for PARP cleavage reveal apoptotic events. Staining with the fluorescent ER-Tracker, MitoTracker, or nuclear stains was similar to nontransfected cells. We thus suggest that cell death may take place due to unspecific effects of E5 expression on cellular membrane compartments rather than due to apoptosis.

Most importantly, we found that the E5 protein colocalizes with Bcl-2 antiapoptotic protein as shown by conventional (Fig. 4) as well as by confocal (Fig. 5) microscopy, although Bcl-2 did not inhibit E5-mediated cell death. All mutant E5 proteins colocalized with Bcl-2 as well. Single transfections with E5 and

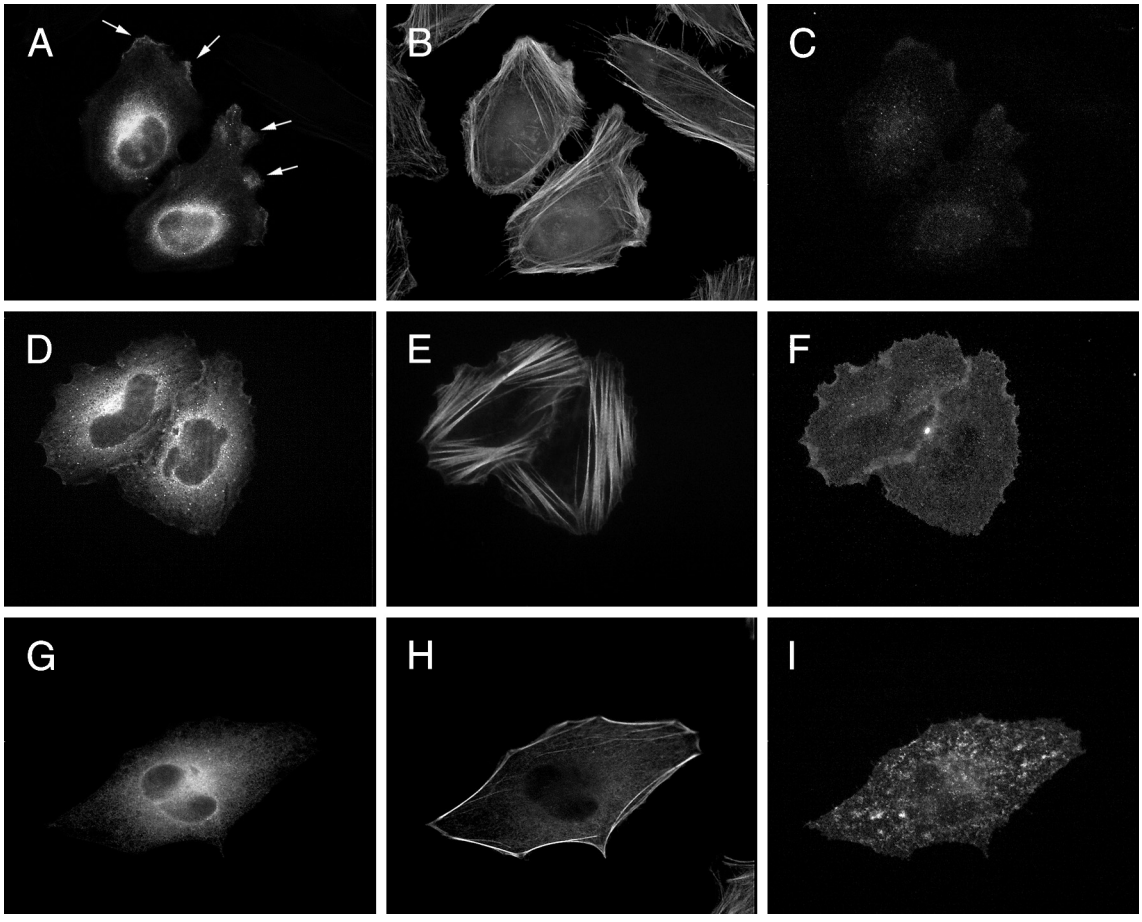

Fig. 3. Cotransfection with M10 (leucine anchor mutant) together with myc-tagged wild-type Rac1 (**A**–**C**), constitutively activated Q61LRac1 (**D**–**F**), and dominant negative T17NRac1 (**G**–**H**). (**A**, **D**, **G**), GFP fluorescence showing M10; (**B**, **E**, **H**), phalloidin staining showing F-actin; (**C**, **F**, **I**), staining with the anti-myc-antibody showing the different Rac1 proteins. Arrows in (**A**) show M10 in lamellipodia

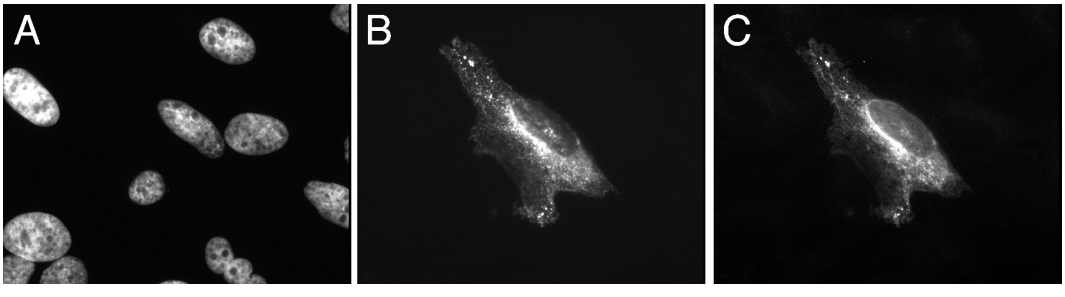

Fig. 4. Colocalization of the E5 protein with Bcl-2. Hoechst nuclear staining (**A**). Transient coexpression of wild-type E5 protein (**B**) and Bcl-2 (**C**) showing identical localization

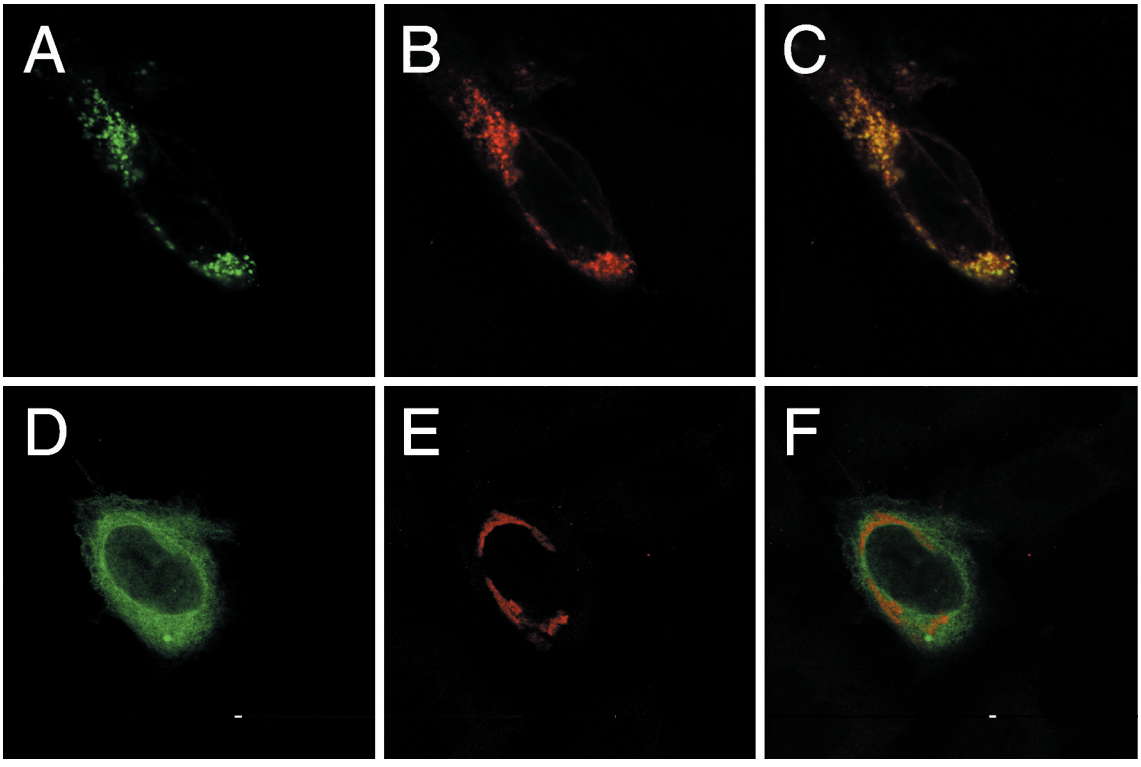

Fig. 5. A confocal image showing colocalization of the E5 protein with Bcl-2 (**A**–**C**), and different localization from Bcl-x_L (**D**–**F**). GFP-E5 protein (**A**), Bcl-2 (**B**), merge (**C**). GFP-E5 protein (**D**), Bcl-x_L (**E**), merge (**F**)

Bcl-2 were performed to control for signal specificity and relocalization of the proteins. It was repeatedly shown that coexpression of E5 and Bcl-2 did not lead to relocalization of those proteins. Another antiapoptotic member of the Bcl-2 family, Bcl-x_L, did not colocalize with E5 (Fig. 5). In preliminary coimmunoprecipitation we were unable to establish direct binding of E5 to Bcl-2.

Effect of the E5 proteins on cell morphology and focal adhesions

No major morphological differences between E5-expressing and control cells were seen in light microscopy. Figure 6 shows typical stress fibers for HeLa epithelial cells in the presence of serum. No remarkable differences in F-actin staining between GFP control, wild-type E5 or mutant E5-transfected cells could be observed. M10 was localized in lamellipodia (arrows in 6G), and a typical cortical actin belt was stained along the cell edges (6H), but M10 expression did not alter the staining pattern for F-actin.

Microtubulus is one of the major components contributing to cell morphology. We stained the E5-expressing cells for β-tubulin but were not able to show alterations caused by E5 expression. Interaction of the cell surface with the extracellular matrix through focal adhesions can be used as a parameter to study altered cellular

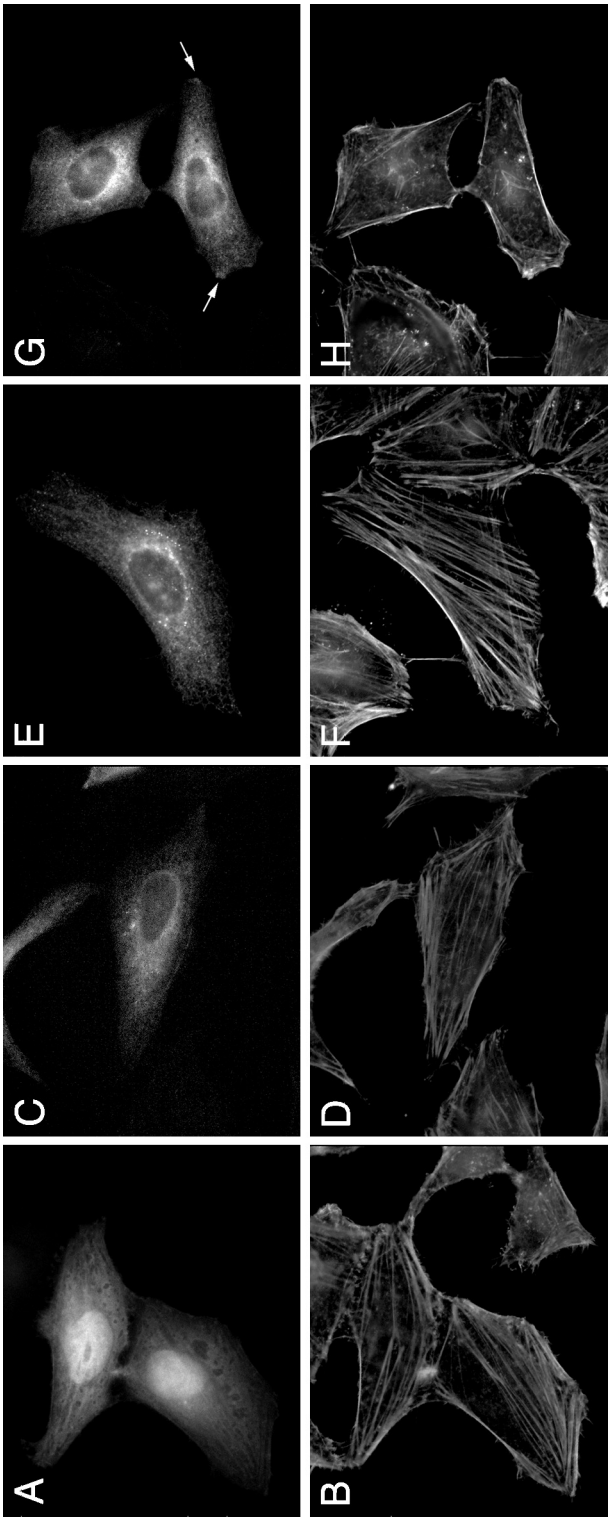

Fig. 6. Actin cytoskeleton in E5-expressing cells. (**A**, **C**, **E**, **G**), GFP fluorescence showing GFP vector control, wild-type E5, M9, and M10; (**B**, **D**, **F**, **H**), phalloidin staining for F-actin. Lamellipodia localization of M10 in (**G**) are shown by arrows

properties [39]. No differences in focal adhesions were seen as studied by staining for paxillin or other focal adhesion components (data not shown).

Discussion

In the present study site-directed mutants of the HPV 16 E5 gene were established in order to study the biological functions of the protein. As many cancers are caused by abnormalities in cell division, migration of cells, and cell adhesion – functions that depend on the cytoskeleton – we wanted to study the effects of E5 expression on cell morphology. In the present study we used HeLa cells, which have been confirmed not to contain the E5 ORF of HPV [32] and can thus be applied to study the morphological effects of the E5 protein. Most importantly, HeLa cells are an excellent model to study cell morphology and changes therein, because they are large and flat, they naturally contain stress fibers, cortical actin, lamellipodia, and filopodia, and these structures are readily formed in HeLa cells upon induction. Due to the lack of E5 antibodies the GFP epitope was used to detect the protein. Interestingly, the small BPV E2 hinge epitope [28] enabled the detection of the E5 protein and thus proved useful in tagging hydrophobic membrane proteins.

A total of eight mutants conserving the spatial demands and putative protein conformation were constructed. All E5 proteins were shown to localize in the ER and Golgi, as has been previously shown by Conrad et al. and by us using mouse COS cells or HaCaT human skin keratinocytes [10, 35]. Some colocalization was found with the 58 K protein, a resident of the Golgi intermediate compartment. Interestingly, Thomsen et al. have shown that the E5 protein changes the morphology of mouse fibroblasts and causes a block in endocytotic trafficking [46]. To our knowledge colocalization of E5 with components of the endocytotic pathway has not been shown. In the present study, staining for antibodies to organelle-specific markers showed that E5-expressing cells contain early endosomes, late endosomes, and lysosomes, respectively, but none of these markers colocalized with E5 fluorescence. Importantly, M10 with leucine-to-lysine mutations at amino acids 22 and 23 in the first putative transmembrane domain revealed a sequence contributing to the intracellular localization of the E5 protein. Disruption of a membrane anchor sequence caused partial relocalization of the protein to the plasma membrane-associated lamellipodia, which are formed in response to the small GTPase Rac1 activity [15, 48]. We showed that inactivation of endogenous Rac1 function abolishes lamellipodia and, consequently, M10 localization. The mechanism for the lamellipodial localization still remains to be elucidated, although the possibility that M10 *per se* would induce lamellipodia formation was excluded.

Immortalized and transformed cells have altered morphology and motility [5, 33]. Earlier reports about E5 function suggest a possible involvement of the actin cytoskeleton. However, we did not observe major morphological effects of the E5 proteins on the actin cytoskeleton despite of careful examination of a large number of cells, although Thomsen et al. reported disruption of the F-actin cytoskeleton in E5-expressing fibroblasts [46]. Microtubulus network and the

components of focal adhesions, such as paxillin, were similar in E5-expressing and control cells. Paxillin binding and disruption of the actin cytoskeleton by the E6 oncogene of bovine papillomavirus has been reported [47], and it was recently shown that paxillin binding is necessary but not sufficient for cellular transformation by E6 [13]. HPV 16 E7 oncoprotein interacts with filamentous actin and represses transcription of fibronectin, an important molecule in cell adhesion and migration [37]. The role of the E5 protein may be to increase the susceptibility of the cells to E6 and E7 function.

We also showed a lethal, non-apoptotic effect of the E5 protein in human cervical epithelial cells when expressed at high levels for several days. We suggest that the E5 protein may interfere with the membrane compartments of the cell and cause cell death by an unspecific mechanism. Transient and low-level expression of the E5 protein seems to take place in natural infections, as suggested by Kell et al. [29], and this would allow cell survival. We cannot exlude the possibility that cells expressing tiny amounts of the E5 protein would have survived. A considerable proportion of codons within the HPV 16 E5 ORF are inefficiently translated in human cells. One could hypothesize that low and/or transient E5 expression would provide an evolutionary advantage, because it would lead to increased cell proliferation, whereas high or long-lasting expression would be lethal. Bible et al. have shown that certain natural variants of the HPV 16 E5 gene associate with neoplasia suggesting that natural mutations within the gene encoding the E5 protein may have an impact [6]. Interestingly, a role for E5 in later stages of viral replication has recently been reported [17, 18].

We find colocalization of the E5 protein with Bcl-2 highly intriguing. The Bcl-2 family consists of proapoptotic and antiapoptotic members, and their net effect on cells seems to depend on a balance between these two opposite functions [36]. Bcl-2 is an antiapoptotic member of the Bcl-2 family, it resides in the ER, mitochondria, and nuclear membranes, and it has recently been shown to fulfill its antiapoptotic function from the ER as well as from mitochondria [45]. Interestingly, both pro- and anti-apoptotic effects of the E5 protein have been reported. Kabsch et al. have reported sensitization of human keratinocytes to osmotic stress-induced apoptosis by the E5 protein [26]. Recently it was published that the HPV 16 E5 protein would protect human primary keratinocytes from apoptosis induced by UV B irradiation [50], or impair TRAIL- or FasL-mediated apoptosis [27]. Although a direct interaction of the E5 protein with the antiapoptotic Bcl-2 protein could not be established in this study, E5 may interfere with Bcl-2 or other members of the family, thus modifying the cellular response to apoptotic signals.

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