Downregulation of Bax mRNA expression and protein stability by the E6 protein of human papillomavirus 16

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Previous studies have shown that human papillomavirus (HPV) 16 E6 inhibits apoptosis induced during terminal differentiation of primary human keratinocytes (PHKs) triggered by serum and calcium. E6 inhibition of apoptosis was accompanied with prolonged expression of Bcl-2 and reduced elevation of Bax levels. In the present study, the effect of E6 on Bax mRNA expression and protein stability was investigated. These studies indicate that stable E6 expression in differentiating keratinocytes reduced the steady-state levels of Bax mRNA and shortened the half-life of Bax protein. These results were confirmed in transiently transfected 293T cells where E6 degraded Bax in a dose-dependent manner. Bax degradation was also exhibited in Saos-2 cells that lack p53, indicating its p53 independence. E6 did not form complexes with Bax and did not induce Bax degradation in vitro under experimental conditions where p53 was degraded. Finally, E6 aa 120–132 were shown to be necessary for Bax destabilization and, more importantly, for abrogating the ability of Bax to induce cellular apoptosis, highlighting the functional consequences of the E6-induced alterations in Bax expression.

INTRODUCTION

Infection of the anogenital mucosa with papillomavirus types referred to as ‘high-risk’ or oncogenic such as human papillomavirus (HPV) 16 and HPV18 is a major factor for the subsequent development of cancer (for review see Zur-Hausen 1996, 2002; Burd, 2003). HPV infection of the basal epithelium perturbs cell differentiation and enhances cell proliferation. Two oncoproteins are encoded by the oncogenic HPVs, E6 and E7, which are consistently expressed in HPV-positive cervical cancers and derived cell lines (Zur-Hausen, 1996).

E6 and E7 contribute to the oncogenic process, at least in part, through their ability to interact with and inactivate key cellular regulatory proteins. E7 associates with tumour suppressor Rb and other cell-cycle regulatory proteins that control cell-cycle progression (Zwerschke & Jansen-Durr, 2000; Munger & Howley, 2002), whereas the E6 oncoprotein interacts with a variety of cellular proteins involved in different signalling pathways, (for review see Thomas et al., 1999; Mantovani & Banks, 2001; Munger & Howley, 2002), of which the best known is p53. HPV E6 binds to p53 via the E6AP protein, a ubiquitin ligase, and induces p53 degradation through the ubiquitin pathway (Scheffner et al., 1990; Huibregtse et al., 1991, 1993). Other targets of E6 include proteins involved in the regulation of transcription and DNA replication, such as p300/CREB-binding protein (Patel et al., 1999; Zimmermann et al., 1999), the transcriptional coactivator ADA3 (Kumar et al., 2002), interferon regulatory factor-3 (Ronco et al., 1998), Gsp2 (Degenhardt & Silverstein, 2001), multicopy maintenance protein 7 (hMcm7) (Kuhne & Banks, 1998; Kukimoto et al., 1998) and the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) (Srivenugopal & Ali-Osman, 2002); signalling components and enzymes such as PKN (Gao et al., 2000) and E6 TP1, (Gao et al., 1999); proteins involved in epithelial organization and differentiation such as paxillin (Tong & Howley, 1997) and E6BP/ERC-55 (Chen et al., 1995); proteins involved in cell–cell adhesion, polarity and proliferation control that contain a PDZ-binding motif, such as the human homologue of the drosophila large tumour suppressor, hDlg (Lee et al., 1997; Kiyono et al., 1997), Scribble (hScrib) (Nakagawa & Huibregtse, 2000), MAGI-1, 2 and 3 (Glaunsinger et al., 2000; Thomas et al., 2002) and Mupp1 (Lee et al., 2000), and proteins involved in apoptosis such as Bak (Thomas & Banks, 1998), c-Myc (Gross-Mesilaty et al., 1998; Veldman et al., 2003) and tumour necrosis factor receptor (TNF R1) (Filippova et al., 2002). Many of the E6 interacting proteins (E6AP, E6TP1, hMcm7, Bak, c-Myc, hScrib, hDlg, MAGI 1-3, Mupp1 Gps2 and MGMT) were described as targets for E6 dependent degradation through the ubiquitin-proteasome.
pathway (for review see Scheffner & Whitaker, 2003). In addition, high-risk HPV E6 proteins can also bind DNA and activate transcription of several genes including the telomerase subunit, hTERT (Oh et al., 2001; Veldman et al., 2001; Gewin & Galloway, 2001).

However, for most of the E6 binding partners, the consequence of interaction with E6, on the virus life cycle or capacity to transform host cell is not well understood.

Squamous epithelial cells are the natural host cells for HPV infection and in vitro cultured genital human keratinocytes have been used to study the effects of HPV E6 and E7 expression. Previous studies have shown that the viral oncoproteins cooperate to immortalize primary human genital keratinocytes (PHKs) and they inhibit keratinocyte terminal differentiation induced by serum and calcium (Schlegel et al., 1988; Hawley-Nelson et al., 1989). We showed previously that HPV16 E6 oncoprotein exhibits two separate biological activities in PHKs. E6 protein by itself is capable of inducing colonies of proliferating cells resistant to serum- and calcium-induced differentiation whereas both E6 and E7 are required for immortalization of PHKs (Sherman & Schlegel, 1996). In further studies, we showed that differentiation of cultured foreskin keratinocytes, triggered by serum and calcium, is a progressive process (2–3 weeks) associated with morphological and biochemical changes characteristic to keratinocyte terminal differentiation in vivo. At the end of this process cell-death with features of apoptosis is observed. Human keratinocyte terminal differentiation was accompanied with time-related changes in the expression of cellular proteins involved in the control pathways of apoptosis including downregulation of Bcl-2 and p53, and upregulation of Bax that coincided with the appearance of morphological signs of apoptosis. E6 expression significantly reduced cell stratification and apoptosis that correlated with prolonged expression of Bcl-2, reduced elevation of Bax and a complete loss of p53 (Alfandari et al., 1999).

Proteins of the Bcl-2 family are important regulators of apoptosis and common targets of viral oncoproteins (for review see Reed, 1998; Tsujimoto & Shimizu, 2000; Thomson, 2001). In squamous epithelium, the expression and topographical distribution of these proteins were shown to be related to stages of differentiation. Bcl-2, a suppressor protein of apoptosis, is expressed exclusively in the undifferentiated basal layer of the epithelium (Hockenbery et al., 1991; Polakowska et al., 1994; Jordan et al., 1996), while expression of Bax, a pro-apoptotic protein, is increased in the suprabasal layers (Jordan et al., 1996; Maruoka et al., 1997; Delehedde et al., 1999). The changes observed in Bcl-2 and Bax expression during serum–calcium differentiation of E6 expressing PHKs (Alfandari et al., 1999) could be a reflection of E6 inhibition of terminal differentiation or the direct effect of E6.

In the present study, the effect of HPV16 E6 on Bax expression and stability was investigated. Bax, a multidomain pro-apoptotic member of the Bcl-2 family, functions in mitochondrial membrane permeabilization and the release of cytochrome c, major events in apoptosis induction (Esposti & Dive, 2003; Scorrano & Korsmeyer, 2003). Bax was described previously as a target of the adenovirus E1B19K (Han et al., 1998), and the EBV Bcl-2 homologue, BALF1, (Marshall et al., 1999).

Results of the present study demonstrate that HPV16 E6 expression reduces both Bax mRNA levels and protein stability in human keratinocytes, and stimulates the degradation of Bax protein in stable and transiently expressing cells. E6 enhancement of Bax degradation was exhibited in Saos-2 cells that lack p53. Using annexin-V binding as a marker for apoptosis, we demonstrate that E6 abrogates Bax-induced apoptosis in transiently transfected cells. Finally, we defined the carboxy-terminal region of E6, spanning aa 120–132, as being essential for E6 function in degradation of Bax and inhibition of Bax-induced apoptosis.

METHODS

Plasmids. The pJS55 expression vectors encoding the epitope-tagged HPV16 E6 and E6 carboxy-terminal truncation mutants were described previously (Sherman & Schlegel, 1996). The pJS55-GFP vector was constructed by cloning the green fluorescence protein (GFP) open reading frame (Chalfie et al., 1994) into pJS55. The p53 expression plasmid pJS55-p53 was described previously (Sherman et al., 1997). Expression plasmids for the human Bax (SFFV-Bax) and Bcl-2 (SFFV-Bcl-2) were kindly provided by S. J. Korsmeyer (Howard Hughes Medical Institute, MA, USA). All SFFV plasmids contain the heterologous promoter from the spleen focus forming virus.

Cell culture, transfection and retroviral infection. PHKs cultured from neonatal foreskins were grown in keratinocyte serum-free medium (K-SFM) supplemented with 5 ng epidermal growth factor ml⁻¹ and 50 μg bovine pituitary extract (Gibco-BRL) ml⁻¹.

Infection of PHKs with retroviral vectors carrying the E6 gene, and selection in G418 (Geneticin), were carried out as described previously (Alfandari et al., 1999). For induction of differentiation, monolayers formed after growth in K-SFM were switched to Dulbecco’s modified Eagle medium (DMEM) containing high-calcium and serum supplemented with 1 μg hydrocortisone ml⁻¹ (Schlegel et al., 1988) and then maintained in this medium for 1–3 weeks. Human 293, 293T and Saos-2 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). Cells were transfected by a modified calcium phosphate procedure as described previously (Sherman & Schlegel, 1996).

Protein degradation assays in vitro and in vivo. In vitro degra-
dation assays with 35S-cysteine-methionine-labelled E6 protein translated in wheat germ extract (WGE) and p53 or Bax proteins translated in reticulocyte cell lysate (RTL) were performed at 25 °C as described previously (Sherman et al., 1997, 2002). For the in vivo assays, cells were co-transfected in duplicates with 2 μg haemagglutinin (HA)-Bax expression plasmid, together with 5–15 μg E6 or mutant E6 plasmid, or vector pJS55 and 2 μg GFP expression plasmid that was used as a control for transfection efficiency. Cells were harvested in RIPA buffer after 48 h and the remaining Bax and GFP levels were determined by immunoblot analysis as described previously (Sherman et al., 2002).
Proteins were detected by autoradiography. In WGE. Proteins were suspended in 300 μl cell lysates in modified RIPA buffer, electrophoresis on polyacrylamide gels and transfer to nitrocellulose membranes were described previously (Alfandari et al., 1999). Filters were cut into strips and reacted with the specific antibodies. Primary antibodies used were as follows: the Bax rabbit polyclonal antibody (SC-493; Santa Cruz Biotechnology) and p53 DO-1 monoclonal antibody (mAb) (SC-126; Santa Cruz Biotechnology), the anti-GFP mAb mixture (Cat. No. 1814460; Boehringer Mannheim), the human involucrin mAb (19018; Sigma), the human actin mAb (1378996; Boehringer Mannheim). Proteins were visualized by enhanced chemiluminescence (ECL) (Amersham), using peroxidase-conjugated anti-mouse IgG (115-035-003; Jackson Immuno Research Laboratories) or protein A-peroxidase (NA9120; Amersham) according to the manufacturer’s instructions. Protein amounts were determined by densitometric scanning (Dinco and Rhenium Biological Imaging System BIS 202). Non-saturated exposures of ECL films were scanned and analysed by using the TINA software.

Co-immunoprecipitation. 35S-labelled Bax or p53 translated in vitro in RTL were mixed with 32P-labelled E6A protein translated in WGE. Proteins were suspended in 300 μl lysis buffer containing 1% NP-40, 100 mM Tris/HC1 (pH 8-0) and 100 mM NaCl (LSAB) or in 300 μl cell lysates prepared from 293T or PHKs using the same buffer. After 2 h incubation with rotary shaking at 4 °C, samples were precleared once with protein A Sepharose and then incubated with anti-Bax or p53 antibody and protein A Sepharose for an additional 2 h with continued rotation. Beads were then collected, washed four times with LSAB and subjected to SDS-12.5% PAGE. Proteins were detected by autoradiography.

RNA isolation and Northern blot analysis. Total RNA was isolated from the keratinocyte cultures by using the RNA–DNA, protein reagent, TriReagent (Molecular Research Center), according to the manufacturer’s protocol. RNA was separated on 1-2% formaldehyde agarose gel, transferred to Hybond-N-filter (Amersham) and detected after hybridization to a 32P labelled random-primed DNA probe. The Bax insert released by EcoRI digest from pSFFV-Bax was purified by gel extraction, and randomly labelled with [x–32P]dCTP by using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Molecular hybridization probes also included a cDNA for human involucrin (0-65 kbp) inserted into pBR322 (Eckert & Green, 1986) and human glyceraldehyde phosphate dehydrogenase. Hybridization was carried out as described previously (Sherman et al., 1988).

Protein stability assay. Cells were incubated with 80 μg cycloheximide ml⁻¹ for the time periods indicated. Cell extracts were obtained by lysis in a modified RIPA buffer. Proteins (100 μg per lane) were analysed by SDS-12.5% PAGE electrophoresis and immunoblotting.

Detection of apoptosis by annexin-V labelling. 293T cells were transfected with the HA-Bax expression plasmid together with E6A, vector or Bcl-2 plasmid. After 24 h, transfected cells were trypsinized, washed once in DMEM/10% FCS and once in PBS and then stained with annexin V-Alexa (Boehringer Mannheim) according to the manufacturer’s instructions.

RESULTS

E6 downregulates Bax mRNA and protein levels during serum–calcium differentiation of foreskin human keratinocytes

We showed previously that in vitro differentiation of PHKs triggered by calcium and serum was accompanied with a gradual decrease in the levels of p53 and an increase of Bax, which was most prominent in the second and third week after serum–calcium switch. Infection of PHKs with a recombinant retrovirus that carries the HPV16 E6 gene resulted in a reduction in the overall levels of p53, and to a lesser extent of Bax. Upon serum–calcium switch p53 levels declined further while Bax levels, although upregulated, were significantly lower than the control (Alfandari et al., 1999). To establish whether E6 affects Bax mRNA expression or protein stability, both parameters were examined in differentiating keratinocytes. Foreskin human keratinocytes were transduced with retroviral vectors carrying the E6 or vector DNA, selected in G418 containing medium and further grown to form monolayers in K-SFM. Transduced keratinocytes were subsequently switched to differentiation medium. Cultures were maintained in this medium for 7–14 days and examined for steady-state levels of p53 and Bax mRNAs, in a Northern blot analysis. Differentiation was monitored by detection of the elevation of involucrin mRNA, hallmark of squamous epithelial differentiation. Representative blot is shown in Fig. 1. Levels of p53 mRNAs were upregulated upon shift to differentiation medium and remained high during the first week and then gradually decreased (Fig. 1a, b, and data not shown). In the E6A virus-infected cultures, the overall levels of p53 mRNA were comparable to or even higher than that of vector cells and the decline was delayed. Levels of p53 mRNAs in E6A expressing cells remained high for up to 2 weeks maintenance in serum–calcium medium. (Fig. 1a, b and data not shown). This is, however, in contrast to, and possibly as compensation for, the markedly reduced levels of p53 protein (detected by Western blot) that reflect the accelerated degradation of p53 protein induced by E6 (Fig. 1c and data not shown).

Bax mRNA is differentially spliced giving rise to different size Bax mRNA species. Bax α mRNA, the size of 1 kb, encodes Bax protein that promotes apoptosis (Bargou et al., 1995). Upon serum–calcium shift Bax α mRNA was downregulated both in vector- and E6A virus-infected cultures. The overall levels of Bax mRNA were lower in E6A as compared with vector virus-infected cultures. This could be due to the degradation of p53 that transcriptionally activates Bax expression (Miyashita et al., 1994; Miyashita & Reed, 1995). The decline observed in Bax mRNA levels was observed in the first or second week after serum–calcium switch (Fig. 1a, b and data not shown). The decline in Bax mRNA preceded the elevation in Bax protein levels normally observed in the vector-infected cultures during the second and third week after serum–calcium switch (Fig. 1c, Alfandari et al., 1999). To determine whether E6 independently affects Bax protein stability, in addition to mRNA expression, the rate of degradation of Bax protein was examined in differentiating keratinocytes after exposure to 80 μg cycloheximide ml⁻¹. Levels of p53 were determined in the same cultures. Representative immunoblot analysis of the protein levels at various time points after addition of cycloheximide are shown in Fig. 2.
As expected, E6A dramatically accelerated the decline of p53 (100-fold reduction as compared with fivefold reduction, in E6A and vector cultures, respectively, 14 h after cycloheximide exposure). The decline in Bax levels in the vector cells was slower than that of p53, yet, significantly accelerated in the E6A-infected cultures (12-fold as compared with twofold decrease in E6A and vector cultures, respectively, after 14 h exposure to cycloheximide).

HPV16 E6 stimulates the degradation of Bax protein in vivo independently of p53

To determine whether E6 is the direct cause for the reduction in stability of Bax protein observed in differentiating keratinocytes, we examined the effect of E6 on Bax expression in transiently transfected 293T cells. Bax expression plasmid that expresses the human HA-Bax protein from the SFFV long terminal repeat, was co-transfected with increasing amounts of DNA of the E6A expression plasmid pJS55-E6A (Sherman & Schlegel, 1996). Transfections were

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carried out together with the pJS55-GFP expression plasmid that served as a control for transfection efficiency. Cells were harvested after 30 h and residual Bax, exogenic and endogenic, was determined by immunoblot analysis with the anti-Bax antibody. Levels of GFP in the same filter were determined by analysis with the anti-GFP antibody. Representative immunoblot is shown in Fig. 3(a). The collated results of Bax protein levels from seven independent assays carried out with the E6A plasmid, are shown in Fig. 3(b). A significant reduction in Bax levels, which correlated to the amounts of transfected E6A DNA, was observed in both exogenic and endogenic Bax. A threefold reduction in the amount of exogenous HA-Bax was measured upon co-transfection of 10 μg E6A DNA with 2 μg HA-Bax DNA. Levels of the endogenous Bax were also reduced in a dose-dependent manner. This is most likely due to the high transfection efficiency into 293T cells. Similar results were obtained when transfections were carried out in 293 cells that lack the SV40 T-Ag (data not shown) or when a plasmid that expresses the HA-tagged E6 protein was used in the transfection assays (Fig. 3c). Co-transfection experiments carried out with the wild-type, non-tagged, E6 expression vector confirmed the ability of the native E6 to stimulate Bax degradation, while a truncated mutant that lacks 31 aa from the carboxy terminus of E6 was defective in this activity (Fig. 3d). Notably, E6 did not enhance the degradation of Bax in vitro, in mixing experiments carried out with 35S-labelled E6A and Bax proteins, under conditions in which p53 was clearly degraded (Fig. 4). These results suggest that conditions that are only present in intact cells, but not in rabbit reticulocyte lysates, may be required for E6-induced degradation of Bax protein.

To evaluate whether enhancement of Bax degradation in E6A transfected cells is associated with p53, co-transfections were carried out in Saos-2 cells, an osteosarcoma cell line that lacks p53. The results shown in Fig. 5 indicate that E6A is capable of reducing Bax protein levels in Saos-2
cells to a similar extent as that observed in 293T cells. This result supports a p53 independent effect of E6 on Bax stability in vivo.

**Activity of HPV16 E6 carboxy-terminal truncation mutants in acceleration of Bax degradation**

To map the amino acid sequences required for HPV16 E6 ability to accelerate Bax degradation in vivo, we analysed a panel of E6 carboxy-terminal truncation mutants, previously defined for their abilities to target p53 for degradation and abrogate p53 transactivation (Sherman et al., 1997). Bax degradation assays were carried out in 293T cells as described above (Fig. 6a and b). Mutant (M) 141 that lacks the 10 carboxy-terminal amino acids exhibited Bax degradation activity comparable to that of full-length E6A. This mutant was previously shown to retain full-length E6A activity in degradation of p53 in vitro.

**Fig. 4.** In vitro degradation assays of p53 and Bax with the E6A protein. 35S-labelled E6A protein synthesized in WGE was incubated with p53 or Bax proteins synthesized in RTL. Incubations were carried out at 25 °C. Samples were removed at the indicated time points and analysed by SDS-PAGE.

**Fig. 5.** E6 stimulates the degradation of Bax in transiently transfected Saos-2 cells. Transfections in Saos-2 cells and protein analyses were carried out as described in Fig. 3. Representative Western blot analysis is shown. Duplicates are marked with bold line.

**Fig. 6.** In vivo Bax degradation with E6A and a series of truncated E6 mutants. 293T cells were transfected with 2 µg DNA of the HA-Bax expression plasmid together with 15 µg DNA of the E6A or truncated mutant plasmids. Transfections were carried out together with 2 µg GFP expression plasmid. Cells were harvested after 30 h and residual Bax levels were determined as described in Fig. 3. (a) Results of representative Western blot analyses carried out with E6A and mutants. (b) Collated results of Bax relative levels obtained from at least four independent transfections for each mutant ±SD. One-way ANOVA was used to obtain statistical significance. E6A, M141 and M132 significantly differ from control in their ability to degrade Bax (P<0.01).
and in vivo (Sherman et al., 1997). This mutant was also capable of producing differentiation resistant colonies in PHKs. M132, which was shown previously to be completely defective in p53 degradation and in the calcium–serum resistance assay, still retained the ability to induce Bax degradation in vivo. These results suggest that E6 ability to induce Bax degradation is independent, at least in part, from E6 ability to degrade p53, compatible with the results described above indicating the ability of E6 to induce Bax degradation in Saos-2 cells. The current study defined, however, aa 120–132 as being essential for E6 mediated degradation of Bax, as mutants M120 and M107 were completely defective in this activity (Fig. 6).

**Examination of physical interaction between HPV16 E6 and Bax**

Although HPV16 E6 lacks the Bcl-2 homology domains that are thought to be important for complex formation among Bcl-2 family members and functional homologues (Reed, 1998), E6 was previously shown to be capable of interacting with Bak and inhibiting Bak-induced apoptosis (Thomas & Banks, 1998, 1999). To examine whether E6 might also bind directly to Bax, we performed co-immunoprecipitation assays by using 35S-labelled in vitro translated proteins (Fig. 7). The HA-tagged Bax protein and E6A protein were translated in RTL and WGE, respectively, proteins were mixed in the absence (Fig. 7a) or presence (Fig. 7b) of unlabelled cell lysates prepared from 293T or PHKs and immunoprecipitated with the anti-Bax antibody. In vitro translated p53 and BMV proteins were used as positive and negative controls, respectively, in these assays. We were unable to detect association between E6A and Bax under experimental conditions that clearly indicated co-immunoprecipitation of E6A with p53, both in the absence or presence of cell lysate (Fig. 7a and b). Pull-down assays carried out with HPV16 GST–E6 fusion protein and in vitro translated Bax or p53 showed similar results (data not shown).

**HPV16 protects cells from Bax-induced apoptosis**

Having shown that E6 induces the degradation of Bax in vivo, we were interested to determine whether E6 could inhibit Bax-induced apoptosis. Ectopic expression of Bax was previously shown to induce apoptosis that was inhibited by Bcl-2 (Kitanaka et al., 1997). Transfection of the Bax expression plasmid, SFFV-HA-Bax, into 293T cells induced apoptosis as detected by annexin-V-labelling and FACS analysis (Fig. 8). Apoptosis induced in 293T cells was dose-dependent and correlated to the amount of transfected Bax DNA (Fig. 8a). Co-transfection of the E6 expression plasmid reduced Bax-induced apoptosis. Both the epitope-tagged E6 protein (E6A) and the native protein (E6) were active in reducing of Bax triggered apoptosis (Fig. 8b and c). To investigate further the correlation between E6 ability to accelerate the degradation of Bax and E6 ability to reduce Bax-induced apoptosis, activities of the E6 truncation mutants, described above, were evaluated in the apoptosis assay. Plasmids that encode the mutant E6 proteins were co-transfected with the SFFV-Bax expression plasmid and apoptosis was assessed by annexin-V labelling and FACS analysis. The SFFV-Bcl-2 plasmid was used as a positive control to show inhibition of Bax apoptosis (Kitanaka et al., 1997). Fig. 8(d) presents the collated results from five independent transfections. Transfection of 5 μg Bax DNA normally induced apoptosis in 35–40 % of the cells. Co-transfection of 15 μg Bcl-2 DNA reduced apoptosis to 15–25 %. To compare the effect of E6 and mutants in independent assays, apoptosis induced by pSFFV-HA-Bax (5 μg) was calculated as 100 % and that induced in the E6/mutant co-transfected cells, was calculated as percentage of the Bax-control. As indicated in Fig. 8(d), co-transfection of Bcl-2 reduced Bax-induced apoptosis to 59 ± 13 % of control. Co-transfection of E6A reduced Bax-induced apoptosis to a similar extent, 65 ± 14 %. A significant, yet lower reduction was obtained by M141 and M132 (74 ± 10 and 80 ± 12 %, respectively).

**Fig. 7.** E6A co-immunoprecipitation assays with p53 and Bax. 35S-labelled E6A protein translated in WGE was mixed with 35S-labelled p53, Bax or brome mosaic virus protein translated in RTL, in the absence (a) or presence (b) of cell extract prepared from 293T or PHKs in LSAB. Immunoprecipitations were carried out with the indicated antibodies. Controls included 5 % of the input proteins (–Ab). Immunoprecipitates were analysed by SDS-PAGE.

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M120 and M107 mutant proteins did not exhibit any protective activity (85 ± 13 and 90 ± 19 %, respectively). These results indicate a correlation between the ability to induce Bax degradation \textit{in vivo} (Fig. 6) and ability to reduce apoptosis induced by Bax.

**DISCUSSION**

Expression of Bax, a pro-apoptotic protein, has been shown to be increased in the suprabasal layers of the skin (Jordan et al., 1996; Maruoka et al., 1997; Delehedde et al., 1999) and in foreskin keratinocytes that were triggered towards differentiation by serum and calcium (Alfandari et al., 1999). Although it is a matter of controversy (Mitra et al., 1997; Gandarillas et al., 1999), it has been suggested that keratinocyte differentiation and apoptosis proceed along the same pathways (Polakowska et al., 1994; Budtz et al., 1994). As replication of HPV occurs in differentiated keratinocytes, in the upper layers of the epidermis, E6 can partially prevent apoptosis by downregulation of the pro-apoptotic members of the Bcl-2 family. Previous studies have shown the ability of E6 to target Bak for degradation (Thomas & Banks, 1998, 1999).

In the present study, we show that HPV16 E6 reduces Bax mRNA expression and protein stability in differentiating human keratinocytes. Bax was shown to be transcriptionally regulated by p53 (Miyashita et al., 1994; Miyashita & Reed, 1995), as well as by c-Myc (Mitchell et al., 2000), both targets of E6 dependent degradation (Scheffner et al., 1990; Gross-Mesilaty et al., 1998). The reduction in Bax mRNA levels could be due to E6-induced degradation of these transcriptional activators. Data from this study and previous data (Alfandari et al., 1999) indicated marked reduction in p53 protein levels in E6 expressing PHKs. Levels of c-Myc were not evaluated in the present study, however, in a recent study carried out with E6 retrovirus-infected PHKs a reduction in Myc protein levels was not detected (Veldman et al., 2003), thus questioning the role of Myc in the E6 effect on Bax transcription. In addition to the effect on Bax mRNA, data described herein indicate that E6 stimulates the degradation of Bax \textit{in vivo}. Enhancement of Bax turnover was demonstrated in half-life determination assays, carried out in PHKs that were switched to differentiation medium and in co-expression assays, in transiently transfected cells, using Bax expression plasmid that directs HA-Bax transcription from a heterologous...
synthesize in vitro binding to Bax is conformation dependent and proteins for the inability to detect complex formation are that E6 and absence of E6, particularly during serum–calcium differentiation of PHKs. Our attempts to detect direct interaction between E6 and proteins that are unstable or destroyed in the procedure were different, reflecting differences in vectors used and cells employed. The mechanism of Bax accelerated degradation by E6 remains to be established. The ubiquitin-proteasome system is involved in the degradation of several of the E6 targets (reviewed by Scheffner & Whitaker, 2003). Binding of E6 to the ubiquitin ligase E6AP, mediates ubiquitination and subsequent degradation of several of the E6 targets. It is not known whether E6AP plays a role in Bax degradation. However, the presence of E6AP, which was supplemented in the reticulocyte lysates, was not sufficient for inducing Bax degradation in vitro, in mixing experiments that were carried out at 25 or 30 °C. Other targets of HPV E6 proteins such as Bak (Thomas & Banks, 1999), Gps2 (Degenhardt & Silverstein, 2001) and ADA3 (Kumar et al., 2002) were also shown to be targeted for degradation by E6 in vivo and not in vitro, although all these proteins were demonstrated to form a complex with E6. The mechanisms that control Bax stability have not been elucidated. Some reports indicated the accumulation of Bax in the presence of proteasome inhibitors, implicating the ubiquitin proteasome system in the control of Bax stability (Chang et al., 1998; Li & Dou, 2000). Other studies, however, failed to show this effect (Dewson et al., 2003). Additional data, point to the possibility that Bax has different rates of proteasome dependent protein degradation in different cell types (Marshansky et al., 2001). Other studies provided evidence on the involvement of a calcium activated calpain in Bax cleavage during apoptosis (Wood et al., 1998; Gao & Dou, 2000).

Data from our recent experiments with the proteasome inhibitor MG 132 and stably transduced normal human keratinocytes, failed to reveal the accumulation of Bax either in the vector or E6 cells upon treatment for 4 h with 10–40 μM of the inhibitor (data not shown). Further studies are needed to reveal the molecular pathways involved in the control of Bax stability in the presence and absence of E6, particularly during serum–calcium differentiation of PHKs.

Our attempts to detect direct interaction between E6 and Bax, have not been successful, consistent with a previous report (Thomas & Banks, 1999). Possible explanations for the inability to detect complex formation are that E6 binding to Bax is conformation dependent and proteins synthesized in vitro lack this conformation or that cellular components that are unstable or destroyed in the procedure of cell extraction are required for binding. Binding of the Adeno 19K protein to Bax was shown to occur upon apoptotic trigger that induces conformational change in Bax and exposure of the amino-terminal domain (Perez & White, 2000). Another possibility is that Bax degradation induced by E6 is indirect and mediated through a yet unknown, cellular component that controls Bax stability. E6 could possibly increase the transcription or stability of this putative protein. Using a series of E6 carboxy-terminal deletion mutants, we defined the region spanning aa 132–120 as being essential for Bax degradation activity. M132 that lacks 19 aa from the carboxy terminus, was still able to induce Bax degradation, while M120 that lacks 32 aa was completely defective in this function. M132 was shown previously to be completely inactive in p53 degradation (Sherman et al., 1997), thus further supporting the p53 independent activity of E6 on Bax protein downregulation.

The mutational analyses carried out in the present study showed correlation between the ability of E6 to induce Bax degradation and ability of E6 to reduce Bax-induced apoptosis, suggesting a potential function for E6 in circumventing cellular signals for apoptosis. This apparently is not the only activity of E6 that is required for the formation of differentiation resistant colonies in PHKs (Schlegel et al., 1988; Sherman & Schlegel, 1996). M132 that showed significant ability to degrade Bax and low, yet consistent activity in protection from Bax-induced apoptosis, was completely defective in its ability to induce differentiation resistant colonies in PHKs. These data suggest that other cellular regulators are involved that contribute to the colony formation activity.

In conclusion, we identified the human pro-apoptotic protein, Bax, as a proteolytic effector of the HPV16 E6 oncoprotein and demonstrated that E6 abrogates Bax function in apoptosis. This provides additional strategy for HPV to prevent cell death in differentiated keratinocytes thereby creating the cellular environment in which HPV late gene expression and assembly can occur.

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