Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types

Miranda Thomas and Lawrence Banks
International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy

Human papillomaviruses (HPVs) are small double-stranded DNA viruses which infect basal keratinocyte stem cells and replicate only in terminally differentiating epithelium. Two of the defining characteristics of terminal differentiation are the cessation of DNA replication and exit from the cell cycle. However, HPVs require the replicative machinery of the host cell to replicate their own DNA and the virus has developed a complex strategy to achieve this. The viral E7 protein can stimulate the degradation of Bak, a pro-apoptotic member of the Bcl-2 family. This report shows that the E6 proteins from HPV-18, HPV-16 and HPV-11 can all bind to Bak in vitro, stimulate its degradation in vivo and reduce Bak-induced apoptosis. However, the non-oncogenic HPV-11 E6 is less effective than the oncogenic E6 proteins in each of these assays, indicating that the ability of HPV to circumvent the apoptosis induced by Bak may contribute to the oncogenic potential of the virus.

Human papillomavirus (HPV) replication occurs in terminally differentiating epithelium, and requires the activation of cellular DNA replication proteins. Unscheduled DNA replication can result in the induction of apoptosis, and the viral E6 protein induces the degradation of p53 to prevent this. It has recently been shown that HPV-18 E6 can also stimulate the degradation of Bak, a pro-apoptotic member of the Bcl-2 family. This report shows that the E6 proteins from HPV-18, HPV-16 and HPV-11 can all bind to Bak in vitro, stimulate its degradation in vivo and reduce Bak-induced apoptosis. However, the non-oncogenic HPV-11 E6 is less effective than the oncogenic E6 proteins in each of these assays, indicating that the ability of HPV to circumvent the apoptosis induced by Bak may contribute to the oncogenic potential of the virus.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses which infect basal keratinocyte stem cells and replicate only in terminally differentiating epithelium. Two of the defining characteristics of terminal differentiation are the cessation of DNA replication and exit from the cell cycle. However, HPVs require the replicative machinery of the host cell to replicate their own DNA and the virus has developed a complex strategy to achieve this. The viral E7 protein can increase the rate of DNA synthesis in keratinocytes without greatly altering the normal pattern of differentiation (Blanton et al., 1992; Cheng et al., 1995). It does this by interacting with a number of important regulatory proteins, including pRb (Dyson et al., 1989), which releases E2F (Chellappan et al., 1992; Pagano et al., 1992; Morris et al., 1993) and upregulates the expression of proliferation-related genes (Phelps et al., 1991; Bandara et al., 1991). This provides the machinery that the virus requires for replicating its own genome. However, these cells are undergoing terminal differentiation and the normal response of such a cell to inappropriate DNA replication is to induce apoptosis. One of the best known inducers of apoptosis is the cellular tumour suppressor, p53, and its activity in HPV-infected cells is circumvented by the viral E6 protein. E6 binds to a cellular ubiquitin–protein ligase, E6-AP, and simultaneously to p53, resulting in the ubiquitin-ation of p53 and its subsequent proteolytic degradation (Werness et al., 1990; Scheffner et al., 1990; Huibregtse et al., 1991, 1993). However, E6 has also been shown to prevent apoptosis in both a p53-dependent and a p53-independent manner (Pan & Griep, 1995). One of the most likely cellular targets of E6, which would explain this p53-independent anti-apoptotic activity, is Bak. We have previously shown that HPV-18 E6 can bind to the Bak protein and stimulate its degradation in vivo (Thomas & Banks, 1998). Bak is a pro-apoptotic member of the Bcl-2 family, a group of proteins involved at a critical point in the control of apoptosis (Reed, 1994; Nagata, 1997, for reviews). These proteins represent ideal targets for virus intervention in apoptosis. Indeed, the adenovirus 19 kDa E1B protein has been shown to act as an anti-apoptotic member of this family (Huang et al., 1997), thus providing another example of the functional similarity between the oncogenic proteins of different DNA tumour viruses.

It has been shown that the abrogation of apoptotic responses is an important factor in the tumorigenicity of many viruses. We were therefore interested to determine whether there was any difference between the oncogenic and non-oncogenic HPV E6 proteins with respect to their interaction with the Bak protein. We have previously shown that HPV-18 E6 is capable of binding with high efficiency to Bak in vitro (Thomas & Banks, 1998). Therefore we first performed GST pulldown assays to determine whether there are any differences in the affinities of different HPV E6 proteins for wild-type Bak in vitro. To do this, GST–18E6, GST–16E6 and GST–11E6 fusion proteins, bound to glutathione resin, were incubated with radiolabelled Bak; GST alone was included as a negative control. The specifically bound proteins were eluted and analysed by SDS–PAGE followed by quantification on a Phosphorimager (Becton Dickinson). Table 1 shows the collated results from several assays. It can clearly be seen that, as expected, Bak binds strongly to 18E6; it also binds, albeit
somewhat less strongly, to 16E6 and to 11E6. In order to assess the specificity of the E6–Bak interaction, in vitro-translated Bax protein was also included and, as can be seen, only background levels of binding were obtained. These results demonstrate that the interaction of E6 with Bak is highly specific, since no binding was seen with the closely related Bax protein. It is also clear from these results that the E6 proteins from both oncogenic and non-oncogenic HPV types can bind to Bak in vitro, although HPV-11 E6 binds least strongly.

We then proceeded to determine whether the E6 proteins from the different HPV types could induce the degradation of Bak in vivo. We have previously shown that the ΔGDBak mutant, which lacks the BH3 death domain and is inactive in apoptosis (Chittenden et al., 1995), is, like the wild-type Bak, susceptible to E6-induced degradation in vivo (Thomas & Banks, 1998). We therefore chose to use this mutant in in vivo degradation assays to obviate the risk of excessive cell death which might be obtained with wild-type Bak. Human 293 cells were transiently transfected with a plasmid expressing HA-tagged ΔGDBak (pCD : HA-ΔGDBak) together with increasing concentrations of plasmids expressing HPV-18 or HPV-16 E6 proteins. After 24 h the cells were harvested and the proteins extracted, as described previously (Thomas & Banks, 1998). The amount of ΔGDBak present was determined by SDS–PAGE followed by Western blotting using an anti-HA antibody; the results of which are shown in Fig. 1(a). The co-transfection of increasing concentrations of HPV-18 E6 results in a concurrent decrease in the amount of ΔGDBak detected; it can also be seen that HPV-16 E6 appears to stimulate the degradation of ΔGDBak to a very similar degree. Since the E6 proteins from non-oncogenic HPV-11 can also bind to Bak in vitro, albeit less strongly, we then wished to investigate whether non-oncogenic HPV E6 could stimulate Bak degradation in vivo. The degradation assay was repeated using increasing concentrations of plasmid expressing HPV-11 E6; HPV-16 E6 was used as a positive control and the results are shown in Fig. 1(b). Interestingly, increasing concentrations of HPV-11 E6 also result in a reduction in the levels of Bak, albeit less effectively than HPV-16 E6. It was possible that this degradation was unrelated to the ability of E6 to interact physically with Bak but was an artefact of the transfection or extraction procedures. We therefore included HA-tagged Bax in the in vivo assay, since we had already shown that it cannot interact significantly with E6 proteins in vitro (Table 1). It can be seen in Fig. 1(b) that HPV-16 E6, which has a strong stimulatory effect upon the degradation of Bak in vivo, has little or no effect upon the levels of Bax protein under identical conditions. It is interesting to note that Bak is highly expressed in the upper epithelial layers but Bax is not (Krajewski et al., 1994, 1996), and therefore there has probably been no selective pressure upon HPV to develop a strategy for Bax control. It will now be of interest to determine whether any other pro-apoptotic Bcl-2 family members are also highly expressed in the epithelium and, if so, whether HPVs have developed any strategy to circumvent them.

We have shown previously that HPV-18 E6 stimulation of Bak degradation is probably dependent upon E6–AP (Thomas & Banks, 1998). We also showed that, unlike the case of p53, the E6–AP protein is involved in the control of Bak levels in the absence of HPV E6 and thus this may represent a method by which the cell normally controls Bak levels (Thomas & Banks, 1998). To verify that the interaction between E6–AP and Bak normally regulates the half-life of the Bak protein, we made stable Saos-2 cell lines expressing either HA-ΔGDBak or HA-ΔCBak, a C-terminal truncation mutant which is unable to bind E6–AP (Thomas & Banks, 1998), and used them to analyse the stability of these two proteins in vivo. We performed a pulse–chase radiolabelling experiment to compare the half-life of the ΔCBak with that of the ΔGDBak mutant used above; the cells were pulse-labelled with [35S]methionine (TranS-Label, ICN) for 1 h, then chased with unlabelled medium for 2 h, samples being taken at 0, 1 and 2 h post-chase. The amount of radiolabel incorporated was analysed by SDS–PAGE and autoradiography and the results are shown in Fig. 1(c). The autoradiograph (upper panel) shows two things: first, that during the 1 h pulse approximately equal amounts of ΔGDBak and ΔCBak have been radiolabelled, indicating that the two proteins are synthesized at similar rates. Secondly, it can be seen that very low levels of radiolabelled ΔGDBak protein remain 1 h post-chase whereas appreciable levels of ΔCBak are detected after 2 h; this indicates that turnover of the ΔCBak protein is considerably slower than that of the ΔGDBak protein. The lower panel of Fig. 1(c) shows a Western blot performed upon a fraction of the 0 h samples, demonstrating the extremely high steady-state levels of the ΔCBak protein, relative to ΔGDBak. The data from several pulse–chase assays were quantified by Phosphorimagery analysis and are represented graphically in Fig. 1(d). It is clear that the half-life of ΔGDBak is less than 1 h, whereas nearly 70% of ΔCBak

<table>
<thead>
<tr>
<th>Resin</th>
<th>Protein</th>
<th>Mean % load retained</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST 18E6</td>
<td>18E6</td>
<td>34.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>GST 16E6</td>
<td>16E6</td>
<td>30.1 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>GST 11E6</td>
<td>11E6</td>
<td>25.3 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Mean of at least five experiments. On GST alone, less than 1% was retained in each case.
remains after 2 h. This difference, extrapolated over a 24 h period, could thus easily result in the comparatively high steady-state levels of ΔCBak demonstrated by Western blotting. This assay demonstrates that the interaction of Bak with E6-AP regulates the turnover of Bak protein in the absence of E6.

Having shown that, like HPV-18 E6, HPV-16 E6 and, to a lesser degree, HPV-11 E6 can induce the degradation of the Bak protein in transiently transfected cells, it was important to show whether they were capable of abrogating Bak-induced apoptosis. To investigate this, 293 cells were transfected with plasmid expressing wild-type Bak, together with plasmids expressing HPV-18 E6, HPV-16 E6 or HPV-11 E6. After 18 h the cells were harvested and an Annexin V-binding assay was performed, as previously described (Thomas & Banks, 1998), using FITC-conjugated Annexin V (Boehringer Mannheim). The cells were subjected to FACS analysis and the results are shown in Fig. 2, where it can be seen that the apoptosis induced by Bak can be abrogated most efficiently by HPV-16 E6, slightly less by HPV-18 E6 and least efficiently by HPV-11 E6. Thus, the ability of HPV E6 to stimulate the degradation of Bak in vivo is not restricted to the oncogenic HPV types, and, in
addition, the efficiency of the E6 protein in stimulating degradation appears to correlate with its effectiveness in reducing the apoptosis induced by Bak. It is notable that the GST–16E6 binds Bak less strongly than GST–18E6 in vitro but is equally efficient in in vivo degradation assays and is more efficient in abrogating apoptosis. This may be a reflection of differences in the stability of the GST fusion proteins or their ability to fold correctly. Alternatively, 16E6 may interact less efficiently than 18E6 with Bak, but more efficiently with other components of the degradation pathway.

Since all HPV types must replicate in similar environments within the differentiating epithelium, it might be asked why there are differences between the oncogenic and non-oncogenic HPV types in their abilities to abrogate both p53- and Bak-induced apoptosis. One possible reason was suggested by the demonstration (Doorbar et al., 1997) that the non-oncogenic HPV types replicate in the lower levels of the stratified epithelium, where the keratinocytes undergo cell division, while the oncogenic HPV types replicate higher in the epithelium, where DNA replication would normally be switched off. It seems probable that such induction of DNA replication would result in a strong apoptotic response, both by p53 and Bak. It has previously been shown that the oncogenic HPV E6 proteins have a stronger effect upon p53 than do the non-oncogenic HPV E6 proteins (Werness et al., 1990; Scheffner et al., 1990). The data presented here indicate that the same is true of the E6–Bak interaction, presumably, at least partly, to circumvent this apoptotic response. Thus, the ability of HPV E6 proteins to abrogate Bak-induced apoptosis reflects their ability to stimulate Bak degradation in vivo. It also reflects the position of replication of oncogenic and non-oncogenic HPV types, providing further evidence to support the suggestion that the ability to circumvent the apoptotic activity of Bak is an important factor in the virus life-cycle.

The authors wish to thank David Pim for useful discussions and comments on the manuscript. The work described here was supported in part by the Associazione Italiana per la Ricerca sul Cancro.

References


Received 5 January 1999; Accepted 2 February 1999