Bovine papillomavirus E7 oncoprotein binds to p600 in naturally occurring equine sarcoids

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Studies regarding the functions of the bovine papillomavirus (BPV) E7 oncoprotein in vivo are lacking and no E7-mediated mechanism underlying mesenchymal carcinogenesis is known. Here, we show that the interaction between the 600 kDa retinoblastoma protein-associated factor (p600) and BPV E7, described in vitro in cultured cells, takes place in vivo in naturally occurring equine sarcoids. In these cancers we detect the expression of E7 and p600, and demonstrate that E7 and p600 co-localize and physically interact. Furthermore, intracellular signals involved in p600 functional activity are found not to be overexpressed, suggesting a different functional activity of p600 in naturally occurring carcinogenesis. Our results demonstrate, for the first time, that E7–p600 interaction occurs during the natural history of BPV-induced equine tumours, suggesting an important role for E7 in carcinogenesis. Finally, the system provides a suitable animal model of papillomavirus-associated cancer to test therapeutic vaccination against E7.

Sarcoids are semi-benign tumours of fibroblastic origin affecting the skin of equids (Nasir & Campo, 2008). Sarcoids very rarely regress, more often persist and can be locally aggressive. Currently, there is no universally effective therapy available for sarcoids (Martí et al., 1993). The pathology of this common equine dermatological neoplasia is not completely understood. Bovine papillomavirus (BPV)-1/2 infection is now recognized as the aetiological factor of sarcoids. Many studies have reported the presence and expression of viral DNA in sarcoids supporting active papillomavirus infection (Chambers et al., 2003; Nasir & Reid, 2006). BPV-1 transforms a variety of rodent cell lines (Dvoretzky et al., 1980) and the transforming activity is due to three viral genes: E5, E6 and E7 (Campo, 2003; Borzacchiello & Roperto, 2008). The BPV-1 E7 protein co-operates with E5 and E6 in inducing cell transformation (Bohl et al., 2001). Mutants lacking the E7 ORF are still able to induce transformation but at a lower efficiency, and produce transformants with altered characteristics (Sarver et al., 1984). It has been recently reported that, in the C127 cell line, BPV-1 E7 interacts with p600, a 600 kDa protein whose basic functions are still to be investigated. DeMasi et al. (2005) have shown that knockdown of p600 reduces the transformation of cells expressing BPV-1 E7. Thus, the transformation activity of E7 is mediated, at least in part, by its ability to interact with p600. Additionally, E7 mutant proteins impair this ability and are transformation defective.

It has also recently been shown that human papillomavirus (HPV) type 16 and type 6 E7 binds to p600 and this binding may correlate with the transformation activity of the viral oncoprotein (Huh et al., 2005), thus suggesting a conserved role for the E7–p600 interaction. The binding between BPV-1 E7 and p600 contributes to cell transformation by inhibiting anoikis. This is a type of apoptosis that is commonly inhibited in cancer cells, allowing them to survive in absence of normal cell–matrix interactions (DeMasi et al., 2007). A major role in anoikis is played by integrin-mediated survival pathways, involving focal adhesion kinase (Fak) signalling. Enhanced Fak signalling may result in cancer development and progression (van Nimwegen & van de Water, 2007), and inhibition of Fak in cultured fibroblasts results in apoptosis (Hungerford et al., 1996).

The interaction of BPV E7 and p600 has not been investigated in naturally occurring tumours and data about the mechanisms underlying E7-mediated oncogenic transformation in vivo are lacking.

To gain insights into the molecular mechanisms of equine sarcoids, three sarcoids (T1–T3) with the normal perilesional skin (normal samples: N1–N3) from the same animals and one normal skin sample from an healthy horse (N4) were investigated. Protein lysates from frozen samples were immunoprecipitated with anti-E7 antibody (kindly provided by Professor P. M. Howley, Harvard Medical School, USA), and analysed by SDS-PAGE and Western blot.

E7 was detected in both sarcoids and perilesional skin (not in normal skin) by immunoprecipitation with the polyclonal antibody anti-E7 followed by Western blot. In all the
samples, the antibody recognized a band of the right molecular mass of similar intensities (Fig. 1a). To further confirm anti-BPV E7 specificity we tested it for immunoprecipitation and Western blot on protein extracts from equine and canine muscular tissues and we did not detect expression of E7.

To further confirm the expression of E7 in sarcoids, the same antibody was used to stain histological sections of equine sarcoid \( (n=9) \). The nine sarcoid samples are known to be positive for BPV-1 DNA. For immunohistochemistry, the anti-BPV E7 was applied at 1 : 1000 overnight and the slides stained as described previously (Borzacchiello et al., 2008).

E7 protein was detected in eight of nine tumours \( (88\%) \). Almost all neoplastic fibroblasts from tumour samples displayed cytoplasmic E7 immunoreactivity. Hyperplastic epidermal epithelium adjacent to the tumour was also stained. We further investigated p600 expression in tumours \( (T1–T3) \) and normal samples \( (N1–N4) \) biochemically and by immunohistochemistry. Equal amount of lysate samples was subjected to SDS-PAGE. The rabbit polyclonal antibody raised against aa 1350–1400 of human p600 \#2 (Bethyl Laboratories) was used for Western blot analysis and the antibody recognized a band of the expected molecular mass in both the three neoplastic tissues and normal skin (Fig. 1b). The blots were stripped and reprobed against mouse anti-actin antibody (Calbiochem) at 1 : 5000 to confirm equal loading of proteins in each lane. Densitometry of autoradiographs was performed by the NIHimage Program and values obtained were normalized to the actin levels and expressed as the densitometric ratio. Expression levels of p600 were increased in neoplastic tissues compared with normal as shown by densitometric analysis (Fig. 1c). An anti-p600 antibody \#1 (Sigma-Aldrich) was used to stain histological sections. Immunohistochemically, seven of nine \( (77\%) \) tumour samples showed positive immunosignal for p600, whereas normal fibroblasts did not stain. Almost all neoplastic cells of tumour samples showed cytoplasmic p600 immunoreactivity. Normal epithelial cells from the epidermis showed membrane p600 staining, according to \textit{in vitro} data (Nakatani et al., 2005). The immunohistochemical and biochemical data indicate that the p600 is overexpressed in cancer samples.

As the p600 protein was present within the cytoplasm of neoplastic fibroblasts where BPV-E7 has also been detected, we wondered whether the two proteins would co-localize. The anti-E7 antibody was used in addition to the anti-p600 antibody \#1 in a double colour immunofluorescence assay as described previously (Borzacchiello et al., 2007). Most neoplastic fibroblasts showed cytoplasmic co-localization of p600 and E7 as judged by the yellow fluorescence of the merged images (Fig. 2a).

In order to evaluate the physical interaction between BPV E7 and p600, we ran co-immunoprecipitation experiments. The immunoprecipitates were probed for the presence of the p600 or E7 by Western blot with the respective antibodies. p600 was detected in E7 immunoprecipitates (Fig. 2b) and, conversely, E7 was detected in p600 immunoprecipitates (Fig. 2c), both in sarcoids \( (T1–T4) \) and perilesional samples \( (N1–N3) \), but not in N4. Our data clearly show that BPV E7 and p600 are present in a stable complex in sarcoids.

Recent evidence suggest that BPV-E7 oncoprotein is involved in anoikis, a type of apoptosis that is induced upon cell detachment by activating integrin-mediated survival pathways, among these the Fak-mitogen-activated protein kinase (Nakatani et al., 2005). To examine this possibility, we determined in p600-expressing tumours, the
expression levels of Fak, p-Fak, p-Mek 1/2 and p-Erk 1/2 by Western blot and immunohistochemistry. Equal amounts of lysate samples were subjected to Western blot and a rabbit polyclonal antibody anti-Fak (Cell Signaling) was used. No differences were noted between cancers and normal perilesional samples (Fig. 3a). To further analyse the activation of Fak, the blots were stripped and reprobed with a rabbit anti-p-Fak (Cell Signaling) antibody that identifies phosphorylated Fak protein at Y397. No differences noticed between cancers and normal tissues (Fig. 3a). The blots were stripped and reprobed with a mouse anti-actin antibody to be sure of equal loading in all the lanes. Immunohistochemically, three of nine (33 %) samples showed positive immunosignal for Fak. It was mostly found within the cytoplasm of neoplastic fibroblasts (Fig. 3b). The Fak immunoreactivity was detected as a strong cytoplasmic signal in one sample and as a weak signal in two sarroid samples. Immunohistochemically, one of nine (11 %) samples showed positive immunosignal for p-Fak. p-Fak was mostly found as a weak signal within the cytoplasm of neoplastic fibroblasts (Fig. 3b). Next, we analysed the expression levels of the phosphorylated form of Erk 1/2, only when activated by dual phosphorylation at Thr 202 and Tyr 204, and its upstream kinase Mek by using phospho-specific antibodies (Cell Signaling) that detect levels of Mek 1/2 only when activated by phosphorylation at Ser 217/221. p-Erk 1/2 and p-Mek 1/2 expression was recorded in cancer and normal samples and no noticeable difference in expression level was revealed among the samples (Fig. 3a). Positive immunosignal for p-Erk 1/2 was found in seven of nine samples (78 %) (Fig. 3b). The p-Erk 1/2 immunoreactivity was scored as strong in six of seven (85 %) samples and moderate in one sample (14 %). Immunohistochemically, five of nine (55 %) samples showed a positive immunosignal for p-Mek 1/2 (Fig. 3b). The immunoreactivity was scored strong in one of five (20 %) samples, as moderate in two samples (40 %), as weak in two sarroid (40 %) samples. p-Erk 1/2 and p-Mek 1/2 expression in equine sarcoids maybe related to different activated pathways.

Few studies have been carried out so far investigating the role of BPV E7 in naturally occurring carcinogenesis. In the present study, we have demonstrated, for the first time, that BPV E7 forms a stable complex with p600 protein during the natural history of BPV-induced equine sarcoids, suggesting a role for this viral oncoprotein in naturally occurring carcinogenesis.

We found E7 expression not only restricted to the sarcoinds, but also in suprabasal and spinous layers of normal epidermis above the tumours. This is in agreement with the recent findings that BPV infection is not confined to the dermis, but also involves the epidermis where viral DNA is
E7 co-operates with E5 in cellular transformation (Bohl et al., 2001), therefore it is possible that E7 may modulate responses of infected epithelial cells to sustain high-level expression of other viral oncoproteins such as E5. p600 has been identified as a binding partner of BPV E7 in different in vitro models, suggesting a role of the complex in transformation (DeMasi et al., 2005). In human foreskin fibroblasts, p600 localizes in the nucleus at close proximity with Rb and, together with clathrin, forms meshwork structures at the leading edges of the membranes being involved in their morphogenesis and cytoskeletal organization (Nakatani et al., 2005). We present data showing, for the first time, expression of p600 in neoplastic fibroblasts in which the protein localizes only in the cytoplasm. Contrary to in vitro studies, we did not find p600 localization in the nuclei, which may reinforce the fact that BPV-1 E7 lacking an Rb-binding domain acts in an Rb-independent manner in vivo (Münger et al., 1989). However, we cannot exclude the fact that a lack of p600 localization in the nuclei maybe due to different systems used (artificial in vitro versus in vivo). More interestingly, our biochemical and immunohistochemical data suggest that p600 is overexpressed in neoplastic fibroblasts, thus suggesting a possible role for this protein in sarcoïd carcinogenesis.

Additionally, as p600 perfectly co-localizes in the same cytoplasmic compartment with E7 and the two proteins co-precipitate whether anti-E7 or anti-p600 antibody is used, we suggest that the complex could play a major role in BPV-induced neoplasia. Taken together they suggest that p600 represents a cellular target for E7 also in vivo and the transformation activity of E7 may be mediated, at least in part, by its ability to bind p600 in naturally occurring equine sarcoïds. We found low expression levels of Fak and p-Fak in p600 expressing sarcoïds. It is possible that p600 in vivo does not activate Fak signalling and/or its functional activity correlates with others factors. We may also speculate that since p-Fak is expressed at low levels in human benign tumours (Owens et al., 1995), it is possible that it also retains this property in sarcoïds, which are non-metastasising lesions.

The importance of the findings demonstrated in this paper lies in the fact that E7 and p600 are able to interact in vivo, although p600 putative downstream signals are not overexpressed in sarcoïds. This is due to the different systems analysed, thus suggesting that the function(s) of p600 should be investigated more in depth in vivo.

Moreover, previous work has shown that several E7 mutants retained the ability to bind p600 being unable to protect C127 cells from anoikis. It is worth noting that the binding between E7 and p600, per se, does not appear to be sufficient for inducing transformation. As a matter of fact, E7 proteins of the 'low risk' HPVs, for which no transforming activity has been described, also bind p600 (Huh et al., 2005). Finally, our investigation into the function of BPV E7 in naturally occurring equine sarcoïds contribute to the understanding of papillomavirus-induced carcinogenesis.

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