

## Short Communication

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# Degradation of hDIg and MAGIs by human papillomavirus E6 is E6-AP-independent

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An important characteristic of the E6 proteins derived from cancer-associated human papillomaviruses (HPVs) is their ability to target cellular proteins for ubiquitin-mediated degradation. Degradation of the p53 tumour suppressor protein by E6 is known to involve the cellular ubiquitin ligase, E6-AP; however, it is presently not known how E6 targets the *Drosophila* discs large (DIg) tumour suppressor and the membrane-associated guanylate kinase inverted (MAGI) family of proteins for degradation. By using an *in vitro* E6-AP immunodepletion assay, these targets were tested for degradation in a E6-AP-dependent manner. The data showed clearly that E6 can direct the degradation of DIg and the MAGI family of proteins in the absence of E6-AP in this *in vitro* system. These results provide compelling evidence for the role of E6-associated ubiquitin ligases other than E6-AP in the degradation of certain E6 targets.

The ubiquitin/26S proteasome pathway is a major route for the selective degradation of eukaryotic proteins. Through the removal of key regulatory components, the pathway helps to control many aspects of cell homeostasis, growth and development. High specificity within the ubiquitin system is achieved by the ubiquitin protein ligase family of E3 enzymes. These enzymes bind to the target substrates, either directly or indirectly, and catalyse the last step in the conjugation process [covalent attachment of ubiquitin to the substrate (Ciechanover, 1998; Ciechanover *et al.*, 2000)]. The first example of a viral protein that uses the proteasome machinery to direct the degradation of a cellular protein was provided by the human papillomavirus (HPV) E6 protein and its stimulation of the ubiquitination and degradation of the cellular tumour suppressor protein p53 (Scheffner *et al.*, 1990; Werness *et al.*, 1990). *In vitro* studies in reticulocyte lysates have shown that the HPV E6 oncoprotein binds to a cellular protein of 100 kDa, termed E6-AP (Huibregtse *et al.*, 1991, 1993), which is the prototype of the HECT (homologous to E6-AP carboxyl terminus) domain-containing ubiquitin ligases (Huibregtse *et al.*, 1995). Under normal circumstances, p53 is regulated via the Mdm2 ubiquitin ligase and E6-AP has no role in this pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). However, in HPV-containing cells, the Mdm2 pathway is bypassed and p53 is degraded constitutively via the E6/E6-AP ubiquitin ligase complex (Scheffner *et al.*, 1993, 1994, 1995; Rolfe *et al.*, 1995; Hengstermann *et al.*, 2001).

Although E6-induced loss of p53 is an important element of E6-induced cellular transformation, recent studies have identified a number of additional cellular targets of E6 that may also play an important role [reviewed by Mantovani & Banks (2001); Thomas *et al.* (2002a)]. The discovery that

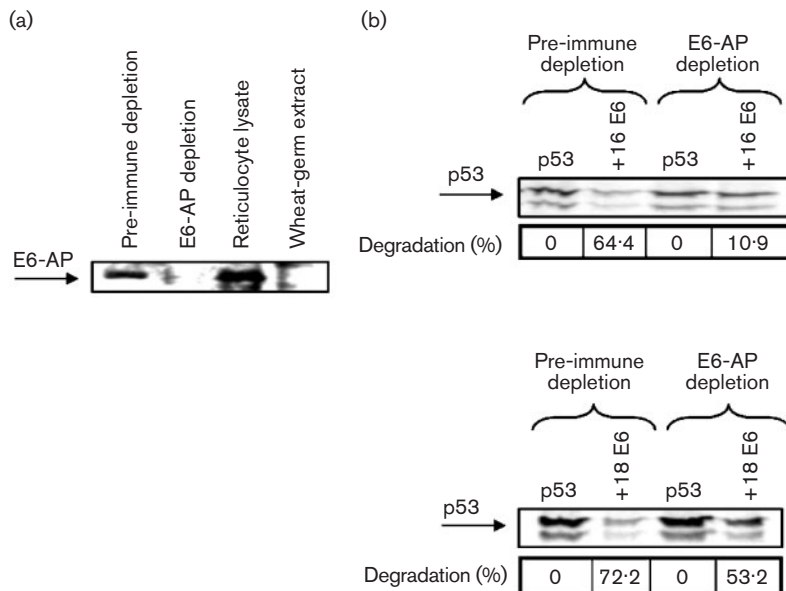
high-risk, but not low-risk, HPV E6 proteins can bind to the PDZ domain-containing proteins and target them for ubiquitin-mediated degradation was particularly interesting, considering that high-risk types cause lesions that can progress to cervical carcinoma, whereas the low-risk types are very rarely associated with malignancies (zur Hausen & Schneider, 1987). PDZ domains are motifs of 80–90 aa, which are present in a variety of proteins that are involved in clustering of ion channels, signalling enzymes and adhesion molecules to specific structures at the membrane-cytoskeleton interface of polarized cells [reviewed by Kim (1997)]. Among them is hScribble, a protein that is expressed at epithelial tight junctions, which has recently been shown to be a substrate for ubiquitination by the E6/E6-AP complex *in vitro* (Nakagawa & Huibregtse, 2000). E6 oncoproteins were also shown to bind to the human homologue of the *Drosophila* discs large (DIg) tumour suppressor (hDIg) and the membrane-associated guanylate kinase (MAGUK) inverted (MAGI) family of proteins and to induce their proteasome-mediated degradation (Gardioli *et al.*, 1999; Glaunsinger *et al.*, 2000; Thomas *et al.*, 2001, 2002b). However, the mechanisms by which these proteins can be directed for degradation by E6 are still unclear (Pim *et al.*, 2000, 2002; Thomas *et al.*, 2001, 2002b).

Recently, we showed that a panel of inhibitory peptides exerted different effects on the ability of HPV E6 to direct the degradation of a number of its substrates. In addition, by using an E6-AP immunodepletion assay, we obtained evidence that MAGI-1 degradation was E6-AP-independent (Sterlinko Grm *et al.*, 2004). Therefore, the present study was initiated to investigate whether E6-directed degradation of the PDZ domain-containing proteins DIg and MAGIs required the E6-AP ubiquitin ligase. We performed *in vitro*

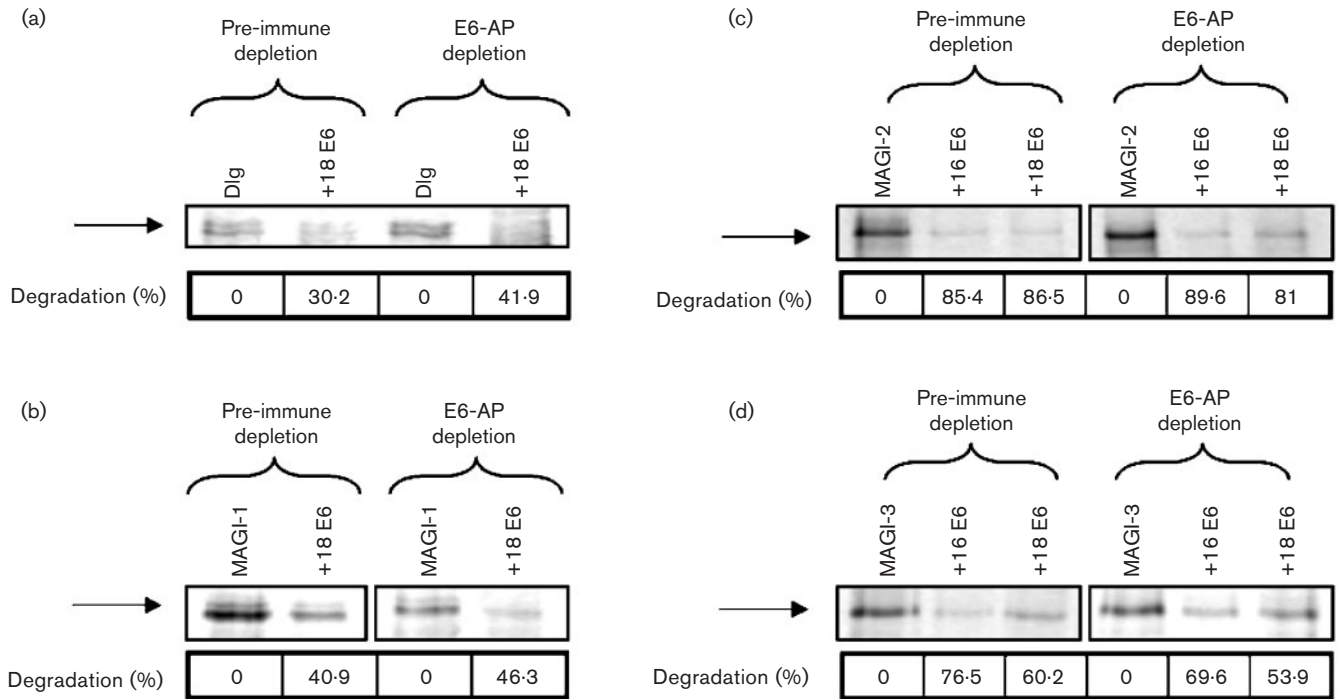
E6-AP immunodepletion assays to determine whether there were any differences in the E6-induced degradation of these targets in the presence or absence of E6-AP. To do this, HPV-16 and -18 E6, p53, hDlg and MAGI proteins were translated *in vitro* by using the TNT rabbit reticulocyte system (Promega) and incubated with rabbit polyclonal anti-E6-AP antibody (kindly provided by Martin Scheffner, Institute of Biochemistry, University of Cologne, Germany) for 30 min on ice, followed by adsorption of the immune complexes onto protein A–Sepharose beads. The supernatant fractions were then used as *in vitro*-translated proteins depleted of E6-AP. Immunodepletion with a pre-immune antibody was included as a negative control. To assess the effectiveness of the depletion, we first performed an anti-E6-AP Western blot on wheat-germ extract (Promega) and on untreated, pre-immune-depleted and E6-AP-depleted rabbit reticulocyte extract (Promega). As shown in Fig. 1(a), no E6-AP was detected in wheat-germ extract, in contrast to rabbit reticulocyte extract, where a strong band was detected at 100 kDa, in agreement with previously published data (Huibregtse *et al.*, 1991). Immunodepletion with anti-E6-AP antibody effectively removed most of the E6-AP protein from the rabbit reticulocyte lysate (Fig. 1a). In contrast, the control immunodepletion with pre-immune sera had little effect. These data showed that the polyclonal anti-E6-AP antibody raised against human E6-AP efficiently recognized the rabbit E6-AP in the rabbit reticulocyte lysate. This is in agreement with the very high degree of conservation in the E6-AP protein between species, with over 99% homology between human and mouse E6-AP. Furthermore, one round of immunodepletion was sufficient to remove most of the E6-AP protein present within the lysate. As it has been demonstrated that E6-mediated degradation of p53 is

E6-AP-dependent, we used this to test the effectiveness of our immunodepletion. *In vitro*-translated p53 was immunodepleted of E6-AP and incubated with similarly immunodepleted HPV-16 or -18 E6 for 30 min at 30 °C. The remaining p53 protein was then immunoprecipitated with rabbit polyclonal C4 antibody and the results obtained are shown in Fig. 1(b). As can be seen, p53 was effectively degraded by E6 over the course of the assay in the sample that was immunodepleted with the pre-immune antibody (negative control). However, E6-mediated degradation of p53 was blocked in the E6-AP-depleted samples, in agreement with previous publications (Huibregtse *et al.*, 1991, 1993; Scheffner *et al.*, 1993).

Having confirmed that by using the E6-AP immunodepletion procedure, we could block E6-mediated degradation of p53 efficiently, we then investigated the role of E6-AP in the E6-mediated degradation of Dlg and the MAGI family of proteins. Lysates containing radiolabelled E6, hDlg and the MAGI proteins were treated as described above and degradation assays were performed. We first investigated the effect of E6-AP immunodepletion on the E6-mediated degradation of Dlg. Assays were performed as for p53, except that the degradation assay required 2 h at 30 °C. After incubation, the amount of residual Dlg was assessed by immunoprecipitation with rabbit polyclonal anti-Dlg antibody (Mantovani *et al.*, 2001). The results obtained are shown in Fig. 2(a). As can be seen, in samples that were immunodepleted of E6-AP, there was the same level of E6-mediated degradation of Dlg as that observed in samples that were immunodepleted with pre-immune antibody, demonstrating that E6-induced degradation of Dlg is indeed E6-AP-independent. We then proceeded to investigate the necessity for E6-AP in the E6-mediated degradation



**Fig. 1.** E6-AP immunodepletion blocks the E6-mediated degradation of p53. (a) Rabbit reticulocyte lysate (10 µl) was mock-depleted by immunoprecipitation with pre-immune antibody (1 µl) or depleted by immunoprecipitation with anti-E6-AP antibody (1 µl). After depletion, residual E6-AP protein was detected by Western blot analysis using rabbit polyclonal E6-AP antibody. Reticulocyte or wheat-germ extracts were included as positive and negative controls, respectively. (b) p53 and HPV-16 and -18 E6 were translated *in vitro* by using the TNT rabbit reticulocyte system (Promega) and depleted with anti-E6-AP or pre-immune (control) antibody. Depleted samples were used in degradation assays. The remaining p53 protein was visualized by immunoprecipitation, followed by SDS-PAGE and autoradiography. Numbers below each lane show percentage degradation of p53, quantified on a Cyclone PhosphorImager (Packard Instrument).



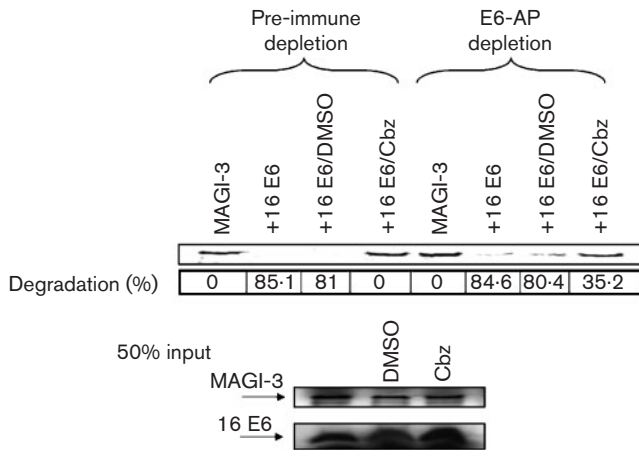
**Fig. 2.** E6-mediated degradation of the PDZ domain-containing proteins Dlg and MAGI-1, -2 and -3 is E6-AP-independent. *In vitro*-translated E6, Dlg and MAGI proteins were subjected to immunodepletion with either anti-E6-AP antibody or a rabbit pre-immune serum. The proteins were then mixed in the combinations indicated and incubated at 30 °C for 2 h for Dlg (a) or 1 h for MAGIs (b–d). The remaining target proteins were ascertained by immunoprecipitation followed by PAGE and autoradiography. Arrows indicate the positions of Dlg and MAGI proteins. Numbers below each panel show percentage degradation of each protein, determined by using a Cyclone PhosphorImager (Packard Instrument).

of three other PDZ domain-containing substrates: MAGI-1, -2 and -3 (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2001, 2002b). MAGI-1, -2 and -3 and HPV-16 and -18 E6 were translated *in vitro* in rabbit reticulocyte lysate, immunodepleted as above and incubated at 30 °C for 60 min. The remaining proteins were immunoprecipitated by using rabbit polyclonal anti-WW antibody (specific for the conserved WW domain on MAGI-1) (Thomas *et al.*, 2002b) and analysed by SDS-PAGE. The results obtained are shown in Fig. 2(b–d). MAGI-1 was degraded to the same extent by HPV-18 E6 over the course of the assay in both the E6-AP- and pre-immune-depleted extracts (Fig. 2b); the same was true for HPV-16 E6-mediated degradation, as demonstrated previously (Sterlinko Grm *et al.*, 2004). The same was also true for MAGI-2 and -3, which were degraded efficiently in E6-AP-depleted extract by both HPV-16 and -18 E6 (Fig. 2c and d, respectively). These results demonstrated that the ability of high-risk E6 proteins to direct the degradation of the PDZ domain-containing targets Dlg and MAGIs does not require the E6-AP ubiquitin ligase.

It has previously been shown that Dlg and MAGI proteins are targeted for ubiquitin-mediated degradation by the high-risk HPV E6 proteins *in vivo* (Gardiol *et al.*, 1999; Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002b). To confirm that E6-mediated degradation of its target proteins in

E6-AP-immunodepleted extracts was through the ubiquitin–proteasome pathway, the degradation assay was repeated in the presence of either the proteasome inhibitor *N*-Cbz-Leu-Leu-Leu-H (Cbz) or DMSO as a control. As shown in Fig. 3 (upper panel), the presence of HPV-16 E6 again resulted in a dramatic decrease in levels of MAGI-3 protein in mock-depleted and E6-AP-depleted extracts. In addition, the proteasome inhibitor Cbz significantly inhibited E6-mediated degradation of MAGI-3 in both extracts, confirming the involvement of the proteasome proteolytic pathway. Cbz treatment also efficiently blocked the degradation of MAGI-1, MAGI-2 and Dlg proteins by both oncoproteins (data not shown).

Previous studies have shown that there are many differences between the E6-mediated degradation of MAGUK proteins and the p53 protein. In contrast to the situation with p53 (Scheffner *et al.*, 1990), HPV-18 E6 binds more strongly to and induces degradation of hDlg and MAGI proteins more efficiently than HPV-16 E6 (Pim *et al.*, 2000). In addition, HPV-18 E6 can induce the degradation of Dlg in wheat-germ extract (Pim *et al.*, 2000), from which E6-AP is absent (Huibregtse *et al.*, 1991, 1993), and extensive mutational analysis of E6 has indicated that sequences within the amino-terminal half of the E6 protein, which are essential for p53 degradation, are largely dispensable for



**Fig. 3.** The proteasome inhibitor Cbz blocks E6-mediated degradation of MAGI-3 *in vitro*. (a)  $^{35}\text{S}$ -labelled MAGI-3 and HPV-16 E6 proteins were translated *in vitro* by using rabbit reticulocyte lysate in the presence or absence of either DMSO or 1 mM Cbz. After pre-immune or E6-AP immunodepletion, the MAGI-3 and E6 proteins were mixed and incubated at 30 °C for 1 h. Remaining MAGI-3 was detected by immunoprecipitation and autoradiography. Numbers below each panel show percentage degradation of each protein obtained, determined by using a Cyclone PhosphorImager (Packard Instrument). The lower panel shows the input of MAGI-3 and E6 protein used in each assay.

the degradation of Dlg (Pim *et al.*, 2000). This broadly covers the region of E6 that is required for high-affinity binding to E6-AP (Li & Coffino, 1996). Lastly, low-risk HPV E6 proteins, which do not interact with E6-AP, can induce the degradation of Dlg when provided with a PDZ-binding consensus sequence (Pim *et al.*, 2000). All of these studies indicate that E6-AP is unlikely to be involved in the degradation of Dlg. In support of this, the data here show clearly that E6 can direct the degradation of Dlg and the MAGI proteins in the absence of the E6-AP ubiquitin ligase in an *in vitro* system. This strongly supports the existence of a second E6-associated ubiquitin ligase, identification of which is now a high priority.

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