Mutational analysis of the discs large tumour suppressor identifies domains responsible for human papillomavirus type 18 E6-mediated degradation

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The discs large (Dlg) tumour suppressor protein is targeted for ubiquitin-mediated degradation by the high-risk human papillomavirus E6 proteins. To understand further the mechanisms behind this, a mutational analysis of Dlg was undertaken. This study demonstrates that an intact PDZ domain 2 (PDZ2) on Dlg is necessary for the ability of E6 to bind and degrade Dlg. However, additional residues within the amino-terminal portion of Dlg are also required for optimal E6 activity. Stable cell lines expressing different Dlg mutants were also established and these confirm that Dlg is regulated intrinsically by the proteasome in the absence of E6; however, in this case, the sequences responsible for regulating Dlg stability lie predominantly within PDZ2. These results suggest that there are at least two mechanisms for regulating Dlg protein stability and that the pathways used by E6 are not necessarily the same as those used in the cell in its absence.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that infect various cutaneous and mucosal epithelial tissues. Those HPVs that infect genital mucosas can be classified as either low or high risk on the basis of their clinical manifestations. The high-risk types, such as HPV-16 and HPV-18, are associated with lesions that can progress to cervical carcinoma (zur Hausen, 1996). The transforming activities of HPV have been shown to reside primarily in the E6 and E7 genes (Vousden, 1994), both of which are retained and expressed continually in cervical cancer-derived cell lines (Smotkin & Wetstein, 1986; Schwarz et al., 1985; Banks et al., 1987; Androphy et al., 1987). The E6 and E7 proteins have been shown to target and interfere with the normal function of key cellular proteins that physiologically regulate cell proliferation and differentiation. The E6 proteins derived from high-risk HPVs have the ability to target some of their cellular partners for ubiquitin-mediated degradation. The first example of this was the p53 tumour suppressor, which, in complex with the E6-AP (E6-associated protein) ubiquitin ligase and the high-risk E6 proteins, is rapidly targeted for ubiquitin-mediated degradation (Scheffner et al., 1990; Huibregtse et al., 1991, 1993). However, there is mounting evidence to suggest that E6 has other oncogenic activities that are independent of its ability to degrade p53 (Pim et al., 1994; Ishiwatari et al., 1994; Inoue et al., 1998; Liu et al., 1999). In support of these studies, E6 has been shown to associate with a large number of other cellular proteins, including Bak (Thomas & Banks, 1998), p300/CBP (Patel et al., 1999), Mcm7 (Kühne & Banks, 1998), E6BP/ERC-55 (Chen et al., 1995), h-Dlg/SAP97 (Lee et al., 1997; Kiyono et al., 1997) and MAGI-1 (Glaunsinger et al., 2000). Many of these other cellular targets regulate vital aspects of cell growth and differentiation.

The human discs large tumour suppressor protein h-Dlg/SAP97 (Dlg) (Lue et al., 1994; Müller et al., 1995), homologous to Drosophila tumour suppressor Dlg-A (Woods & Bryant, 1991), is a member of the MAGUK (membrane-associated guanylate kinase homologous) family of proteins. These proteins are characterized by having specific protein recognition domains, including SH3, PDZ and guanylate kinase homologous (GuK) regions (Anderson, 1996). PDZ domains are specific modules for protein–protein interactions (Ponting & Phillips, 1995; Fanning & Anderson, 1999) that allow the clustering of proteins and the formation of...
multiprotein signalling complexes at specialized sites in the membrane (Kim, 1997). For protein interaction with PDZ domains, a carboxy-terminal S/TVX motif is required in the partner molecules (Songyang et al., 1997). In epithelial cells, Dlg is co-localized with E-cadherin at sites of cell–cell interaction (Reuver & Garner, 1998), where it is thought to have both structural and signalling roles. Epithelia form structures of polarized cells with the apical and basolateral sides separated by cell junctions and Dlg is required for this organization (Kim, 1997). Deregression of this system leads to defective cell–cell adhesion, loss of cell polarity, unregulated proliferation and alteration of the pattern of cell differentiation (Woods et al., 1996). Dlg has been shown to bind via its PDZ domains to the carboxy terminus of the APC (adenomatous polyposis coli) tumour suppressor (Matsumine et al., 1996) and it was shown that the APC–Dlg complex is important for APC-mediated growth suppression (Ishidate et al., 2000). Recent studies have shown that in Drosophila, Dlg and two other tumour suppressors, scribble and lgl, act cooperatively to regulate cell polarity and proliferation (Bilder et al., 2000). This finding suggests an important connection between epithelial organization and cellular growth control and points out the critical role of these oncosuppressors in this regulatory system (Bilder et al., 2000). In addition, Dlg is a target for several viral oncoproteins, including human T cell leukaemia virus type 1 Tax, high-risk HPV E6 proteins and adenovirus type 9 E4-ORF1 protein (Lee et al., 1996; Goode & Perrimon, 1997).

We have shown previously that the binding of HPV-16 and -18 E6 proteins to Dlg results in a dramatic reduction in the levels of Dlg both in vivo and in vitro and this is mediated by the ubiquitin proteolytic pathway (Gardiol et al., 1999). We have shown also that this activity of E6 is regulated specifically by protein kinase A phosphorylation of the HPV E6 carboxy-terminal motif, which is involved in the binding to the PDZ domains (Kühne et al., 2000). To investigate further this regulation of Dlg by ubiquitinylation, we constructed a series of Dlg mutants and investigated their susceptibility to proteasome-mediated degradation in the presence and absence of HPV E6. We show that PDZ domain 2 (PDZ2) of Dlg is necessary for the ability of E6 to target Dlg for degradation and sequences within the extreme amino-terminal region of Dlg (NT), prior to the first PDZ domain, are also required for optimal degradation efficiency. In the absence of E6, Dlg is also subjected to ubiquitin-mediated degradation, but, in this case, all of the sequences required would appear to reside within PDZ2. Sequences within the carboxy-terminal SH3 and GuK domains do not appear to be involved in proteasome-mediated regulation of Dlg.

**Methods**

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**Plasmid constructions.** The rat SAP97 (Dlg)-deleted mutant derivatives (Fig. 1) were constructed by PCR using pairs of primers flanking the region to be amplified, according to the reported sequence for Dlg and using pGEX-2T-DLG as template DNA (Mueller et al., 1995; Lee et al., 1997). PCR products were cloned in-frame into the HA-tagged epitope. The h-Dlg/SAP97 used as template DNA for PCR amplification corresponds to the reported IS3 Dlg isoform (Müller et al., 1995). NT refers to the amino-terminal region of the Dlg protein prior to the first PDZ domain. Dlg-NTPDZ1 mutant, aa $1–276$ of the reported sequence; Dlg-NTPDZ1-2, aa $1–382$; Dlg-3PDZ, aa $186–511$; and Dlg-NTPDZ3, aa $1–185$ in-frame with aa $433–511$, indicating the deletion of PDZ1 and -2. Mutants DLG-ΔNT and DLG-ΔSH3 bear deletions encompassing aa $1–185$ and aa $549–617$, respectively.
products were cloned into the HA-tagged expression vector in a three pieces ligation. Constructs, confirmed by partial sequence analysis, were used for the in vitro and in vivo expression of Dlg proteins.

The cloning of HPV-18 E6 into pSP64 for in vitro translation, pCDNA-3 for in vivo expression and pGEX-2T for the expression of glutathione S-transferase (GST) protein fusion in bacteria has been described previously (Thomas et al., 1995).

- **Cells and tissue culture.** Human 293 and U2OS cells were grown in DMEM supplemented with 10% foetal calf serum. Transient transfections were carried out using the calcium–phosphate precipitation method described previously (Matlashewski et al., 1987). Transfection efficiencies were tested by co-transfecting the *Escherichia coli* β-galactosidase-expressing plasmid pCH100 in parallel and assaying for β-galactosidase activity.

  U2OS cells stably expressing HA-tagged Dlg proteins were selected and maintained in culture medium supplemented with geneticin antibiotic (G418) at a concentration of 200 μg/ml.

- **GST fusion protein expression and binding assays.** GST–HPV-18 E6 fusion protein was expressed in *E. coli* and purified on glutathione–agarose beads. GST pull-down assays were performed as described previously (Thomas et al., 1995).

- **In vitro and in vivo degradation assays.** In vitro degradation assays were performed as described previously (Pim et al., 1994). Briefly, in vitro-translated HA-tagged Dlg proteins were mixed with in vitro-translated wild-type HPV-18 E6 or water-primed lysates as a control at 30 °C. At the indicated time-points, reactions were immunoprecipitated using an anti-HA antibody (Boehringer Mannheim) and the remaining Dlg proteins were visualized by autoradiography after SDS–PAGE resolution.

  For in vivo degradation experiments, cells were harvested in extraction buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES pH 7.0 and 1% aprotinin) 24 h after transfection. Equal amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes. Levels of recovered Dlg protein were determined by immunoblotting using an anti-HA monoclonal antibody (mAb) and the blots were developed using ECL, according to the manufacturer’s instructions (Amersham).

  For proteasome inhibitor protection assays, cells stably expressing different Dlg mutants were treated with either 50 μM N-CBZ–LEU–LEU–LEU–AL proteasome inhibitor or an equal amount of DMSO as a control 2 h prior to protein extraction. Levels of Dlg protein were then ascertained by immunoblotting, as described above.

### Results

#### Identification of the domains of Dlg required for E6-mediated degradation in vitro

Previous studies had shown that reduced levels of Dlg protein in the presence of HPV-18 E6 correlate with the ability of the two proteins to form a complex. This binding involves the carboxy-terminal domain of the E6 protein (XS/TVX) and at least one of the three PDZ domains of Dlg (Gardiol et al., 1999). We showed that a Dlg derivative, containing the amino terminus and the three PDZ domains (DLG-NT-3PDZ), contains all of the sequences necessary and sufficient for E6-mediated degradation (Gardiol et al., 1999). To investigate further the precise regions of Dlg required for E6-mediated degradation, a series of Dlg-deleted mutants within the key domains of the protein were produced; these are shown schematically in Fig. 1. To investigate the susceptibility of these mutant Dlg proteins to E6-mediated degradation, we carried out a series of in vitro degradation assays. The HA-tagged Dlg proteins were in vitro-translated with rabbit reticulocyte lysate and incubated in the presence of in vitro-translated E6 at 30 °C for 1 and 2 h and residual Dlg was measured by immunoprecipitation, SDS–PAGE and autoradiography. The results obtained are shown in Fig. 2. As can be seen, deletion of the amino-terminal amino acids prior to the PDZ domain of Dlg reduces its susceptibility to E6-induced degradation when compared with the wild-type protein, since
its rate of degradation is significantly slower (Fig. 2A). In contrast, deletion of the SH3 domain has no effect on the susceptibility of Dlg to E6-induced degradation, while the portion of Dlg encompassing just the three PDZ domains is degraded in a manner similar to that protein lacking the amino-terminal region (Fig. 2B). Finally, the removal of PDZ2 abrogates the ability of E6 to degrade Dlg, where both the DLG-NTPDZ1 and DLG-NTPDZ3 proteins are resistant to E6-induced degradation (Fig. 2, C and D). However, DLG-NTPDZ1-2 is degraded efficiently by E6, although to a somewhat lesser extent than the wild-type (Fig. 2C). These results suggest therefore that there are two regions of Dlg that contribute to its susceptibility to be degraded by E6: PDZ2 and the amino-terminal amino acids prior to PDZ1.

Since E6 has been shown to interact with Dlg via one or more PDZ domains, we investigated whether the differences observed between the Dlg mutants in the degradation assay were related to E6-binding activity. In order to do this, we performed a GST pull-down assay using GST–18E6 and the in vitro-translated radiolabelled Dlg mutants. The results of a representative experiment are shown in Fig. 3. As can be seen, DLG-NTPDZ1-2, DLG-3PDZ, DLG-∆NT and DLG-∆SH3 are all bound by HPV–18 E6 in a manner similar to wild-type Dlg.

In contrast, DLG-NTPDZ1 and DLG-NTPDZ3 completely fail to bind to HPV–18 E6 in this assay. These results demonstrate that HPV–18 E6 principally recognizes Dlg via PDZ2, in agreement with previous observations for HPV–16 E6 (Kiyono et al., 1997) and thereby explains the inability of HPV–18 E6 to target the DLG-NTPDZ1 and DLG-NTPDZ3 mutants for degradation. In contrast, the reduced levels of degradation of the mutants of Dlg lacking the amino-terminal amino acids are not due to reduced levels of E6 binding and suggest that this region of Dlg is also required for optimal susceptibility to E6-induced degradation.

**Susceptibility of the Dlg mutants to proteasome-mediated degradation in vivo**

Having determined which regions of Dlg were required for E6-mediated degradation in vitro, we wished to assess them in vivo, since previous studies had highlighted potential differences between the two assay systems (Foster et al., 1994; Gardiol & Banks, 1998). U2OS cells were co-transfected with the HA-tagged Dlg-expressing plasmids and wild-type HPV–18 E6 or vector alone. After 24 h, the cells were harvested and the levels of Dlg protein were determined by Western Blot using an anti-HA mAb. The results obtained are shown in Fig. 4. As can be seen, mutants DLG-NTPDZ1-2 and DLG-∆SH3 are degraded with wild-type efficiency and mutant DLG-NTPDZ1 is largely resistant to E6-induced degradation. Interestingly, however, mutants DLG-3PDZ and DLG-∆NT also appear to be much less susceptible to E6-induced degradation. Identical results were also obtained when the assay was performed using 293 cells, suggesting that these observations are not cell type-dependent. These results demonstrate therefore that, in addition to the presence of PDZ2, sequences within the extreme amino-terminal region of Dlg would also appear to be required for an efficient E6-mediated degradation in vivo as well as in vitro.

**Regulation of Dlg by proteolysis in the absence of E6 in vivo**

We showed previously that Dlg was a target for ubiquitin-mediated degradation even in the absence of the E6 viral protein, suggesting that this may be the normal physiological way of regulating Dlg activity in the cell. In addition, we showed that the Dlg derivative mutant DLG-NT-3PDZ contains all of the sequences necessary and sufficient for this process (Gardiol et al., 1999). To characterize further the regions of Dlg that confer susceptibility to proteasome regulation in the absence of E6, we generated cell lines expressing three of the HA-tagged Dlg mutant proteins, NTPDZ1, NTPDZ1-2 and 3PDZ. The levels of expression of the three mutant Dlg proteins were then assessed by Western blotting using an anti-HA mAb. Prior to protein extraction, the cells were also treated for 2 h with either the proteasome
Dlg domains needed for degradation by HPV E6

Fig. 4. Degradation of Dlg proteins mediated by HPV-18 E6 in vivo. U2OS cells were transfected with 5 µg HA-tagged Dlg-expressing plasmid and 5 µg HPV-18 E6-expressing vector or 5 µg vector alone as indicated. For the DLG-NT degradation assay, 1 or 5 µg of E6 expression plasmid was used as indicated. After 24 h, cell proteins were extracted and equal amounts were separated by SDS–PAGE. Levels of Dlg protein were determined by Western blot using an anti-HA mAb.

Fig. 5. Regulation of Dlg stability in the absence of E6. U2OS cells were stably transfected with either pCDNA-3 alone (C) or the indicated Dlg mutant derivatives. Cell lines were then incubated in the presence (+) or absence (−) of the proteasome inhibitor CBZ for 2 h. After this time, cells were harvested and levels of Dlg mutant protein expression determined by Western blotting using an anti-HA mAb. The positions of the respective mutants are indicated by arrows.

inhibitor CBZ or DMSO as a control. The results obtained are shown in Fig. 5. As can be seen, proteasome inhibition results in a very strong stabilization of the NTPDZ1-2 and 3PDZ mutant proteins. In contrast, the level of stabilization of the NT-PDZ1 mutant is much less when compared with the mutants that retain an intact PDZ2. These results demonstrate that the principal sequences necessary for proteasome-mediated regulation of Dlg, in the absence of E6, lie within PDZ2.

Discussion

Dlg is intimately involved in the regulation of cell adhesion and cell polarity. As such, the degradation of Dlg by E6 oncoproteins may have important implications for the development of cervical cancer (Lee et al., 1997; Kiyono et al., 1997; Gardiol et al., 1999; Pim et al., 2000). E6 oncoproteins were demonstrated to target other PDZ domain-containing proteins, such as MAGI-1, MUPP-1 and the human homologue of the Drosophila scribble oncosuppressor, h-scrib (Glaunsinger et al., 2000; Lee et al., 2000; Nakagawa & Huibregtse, 2000). h-scrib is associated also with cell junctions and it co-operates with Dlg-regulating epithelial polarization and cell proliferation (Bilder et al., 2000). The ability of E6 to degrade both proteins with similar cellular functions emphasizes the relevance of these activities of E6 oncoproteins in HPV-associated carcinogenesis. Since E6 makes use of a pre-existing proteolytic pathway, a better understanding of the mechanisms by which Dlg protein levels are regulated is important for understanding the function of E6, as well as the mechanisms by which Dlg is controlled normally. In order to do this, we generated a series of Dlg deletion mutants to determine the regions of Dlg required for proteasome-mediated degradation. By performing a series of in vitro and in vivo E6-mediated degradation assays, we showed that an intact PDZ2 on Dlg was necessary for the ability of E6 to target Dlg for degradation. By doing a series of GST pull-down assays, we found that this activity of E6 correlated with the ability of E6 to bind Dlg. This suggests that PDZ2 may be the principal binding site of HPV-18 E6 on Dlg and is in agreement with previous studies on HPV-16 E6 (Kiyono et al., 1997). Interestingly, however, we found that sequences within the extreme amino-terminal region of Dlg, prior to PDZ1, were also required for the full efficiency of this system. Thus, although DLG-3PDZ and DLG-NT were degraded by E6, they were significantly less susceptible in vivo and both mutant proteins lacking the amino-terminal region were degraded at a much slower rate than wild-type Dlg in vitro. Since we showed that this amino-terminal region of Dlg is not involved in binding E6 (Gardiol et al., 1999), this result suggests that this region may be required for efficient binding of the cellular ubiquitin ligase or other components of the degradation machinery. The specific ubiquitin ligase for Dlg has not been identified yet, but it has been shown that E6-AP is probably not involved in this system (Pim et al., 2000). It is worth noting, however, that among the synaptic associated proteins (SAPs) that belong to the MAGUK family of proteins, this amino-terminal region is unique for each protein and, in this way, may contribute to the unique features of each SAP. In the case of h-Dlg/SAP97, it has been shown that the first 65 amino acids, which are absent in other SAPs, direct the selective subcellular localization of the protein at regions of
cell–cell contact and mediate attachment to the cytoskeleton (Wu et al., 1998). The fact that this region would appear to be required for E6-mediated degradation in vivo also raises the possibility that incorrect localization within the cell may render the DLG-3PDZ and DLG-ANT mutants less susceptible to E6-mediated degradation.

Having determined which regions of Dlg were required for E6-mediated degradation, we were next interested in investigating whether those same regions were involved in regulating Dlg protein stability in the absence of E6. This was particularly interesting, since we had shown previously that wild-type Dlg was regulated intrinsically by the proteasome in the absence of E6 (Gardiol et al., 1999). To address this question, we generated a series of cell lines stably expressing some of the Dlg deletion mutant proteins and then asked whether they could be stabilized following treatment of the cells with proteasome inhibitors. The reasoning being, that if any mutant was processed by the proteasome, then blocking its activity should give rise to an increase in the steady-state levels of the mutant protein. Interestingly, DLG-NTPDZ1-2 and DLG-3PDZ both showed strong increases in protein levels following proteasome inhibition, suggesting that both proteins were being degraded by the proteasome. In contrast, DLG-NTPDZ1 was largely unaffected by this treatment and was only weakly stabilized when compared with the other mutants, suggesting that the protein levels of this mutant Dlg are probably not being regulated by the proteasome. Unfortunately, this mutant is expressed at quite low levels within the stable cell lines, which runs counter to the above argument. However, it is possible that within the context of a stable cell line, this mutant may have inhibitory effects on cell growth and, hence, only be tolerated at low levels. Further studies will be required to clarify this issue. The above results raise a number of interesting points. As in the case of E6, the contribution of PDZ2 to the regulation of Dlg levels in the absence of E6 appears to be essential. However, unlike the case with E6, the amino-terminal region of Dlg does not appear to be important for this regulation in the absence of E6. Therefore, it seems that, as for other cellular targets of E6, different proteins or pathways are involved in the proteolytic degradation of Dlg in the absence or presence of E6. Considering the central role of PDZ2 in regulating Dlg protein stability in the absence of E6, it is interesting also to note that the APC tumour suppressor has been shown to interact with this domain of Dlg and this raises the intriguing possibility that occupation of PDZ2 by APC may contribute to the regulation of Dlg levels and activity. A recent study reported the finding of a PDZ-binding kinase (PBK) which is cell cycle regulated by phosphorylation at mitosis. PBK binds to PDZ2 of Dlg and could probably link Dlg to signal transduction pathways regulating cell cycle and proliferation (Gaudet et al., 2000). This finding emphasizes the importance of PDZ2 in the modulation of Dlg activities and it is striking that it is this domain that is targeted by the high-risk HPV E6 proteins.

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