Proteasome-mediated regulation of the hDlg tumour suppressor protein

Fiamma Mantovani, Paola Massimi and Lawrence Banks*

International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy

*Author for correspondence (e-mail: banks@icgeb.trieste.it)

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SUMMARY

The Dlg tumour suppressor protein is intimately involved in the control of cell contact and polarity. Previous studies have shown that hDlg is a target for a number of viral transforming proteins. In particular, the high risk human papillomavirus (HPV) E6 proteins target hDlg for proteasome-mediated degradation, an activity that appears to contribute to HPV-induced malignancy. However, little information is available concerning the normal regulation of hDlg. In this study we have investigated the role of the proteasome in the regulation of endogenous hDlg protein levels in epithelial cell lines. We demonstrate that hDlg is, indeed, degraded via the proteasome both in the presence and absence of HPV, in a fashion that is dependent on the ability of the cells to form cell junctions. By western blot and immunofluorescence analysis we show that hDlg is efficiently degraded in isolated cells; however, upon cell-cell contact, hDlg is both hyper-phosphorylated and stabilised. Strikingly, in both transformed rodent cells and undifferentiated cervical cancer cells, this ability to stabilise Dlg upon increased cell density is lost. These results demonstrate a complex pattern of hDlg regulation by phosphorylation and proteasome degradation in response to cell contact. Loss of this regulation probably represents a significant step in the development of malignancy.

Key words: Dlg, Proteasome, Transformation, HPV

INTRODUCTION

Epithelia are organised in sheets of specialised cells that are connected by various types of junctions. Such intercellular contacts are crucial for maintaining cell adhesion, cell polarity, cytoskeletal structure and for regulating cell proliferation: during carcinogenesis, loss of these characteristics leads to tissue disorganisation and progression into metastasis. Recently, studies using *Drosophila* have demonstrated that a group of tumour suppressor genes encoding membrane-associated proteins cooperate in the same pathway to regulate both epithelial structure and cell proliferation (Bilder and Perrimon, 2000; Bilder et al., 2000). Two of these, Dlg and Scrib, are multidomain proteins containing PDZ motifs, sites of protein-protein interaction involved in the clustering of ion channels, signalling enzymes and adhesion molecules for specific cytoskeletal structures found at the membranes of polarised cells (Craven and Bredt, 1998; Kim, 1997). *Drosophila* Dlg has long been known to be involved in cell growth control, maintenance of cell adhesion and cell polarity in both embryonic and adult tissues (Woods and Bryant, 1991; Woods and Bryant, 1993; Woods et al., 1996) and in blocking cell invasion during development (Woods et al., 1996). Its closest human homologue is hDlg, a peripheral membrane protein expressed in a variety of cell types, including epithelia (Lue et al., 1994). In these cells, hDlg associates with the cortical cytoskeleton that underlies the plasma membrane at cell-cell adhesion sites (Lue et al., 1996; Wu et al., 1998; Reuver and Garner, 1998). Specific PDZ domains of hDlg have been shown to interact with the C-termini of several proteins including Shaker-type K^+ channels (Kim et al., 1995), cytoskeletal protein 4.1 (Lue et al., 1994; Marfatia et al., 1996) and the APC tumour suppressor protein (Matsumine et al., 1996). Moreover, it contains a proline-rich N-terminal domain with two potential SH3-domain-binding sites (Lue et al., 1994; Ren et al., 1993), which could allow it to participate in signalling pathways by forming complexes via the SH3 domains of other proteins. As it is recruited to cell junctions by E-cadherin-mediated cell adhesion (Reuver and Garner, 1998) and forms a complex with APC that inhibits proliferation by blocking cell cycle progression (Ishidate et al., 2000), it is likely that hDlg will perform functions similar to its *Drosophila* homologue, playing an intimate role in the processes that regulate cell polarity and proliferation in response to cell contact in epithelial cells.

In line with its role as a potential tumour suppressor, hDlg has also been shown to interact, through its PDZ domains, with several viral oncoproteins, including Adenovirus 9 E4ORF1 protein, HTLV-1 Tax and the high risk HPV E6 proteins. These interactions interfere with hDlg binding to APC and can perturb cell growth control (Kiyono et al., 1997; Lee et al., 1997; Suzuki et al., 1999). Significantly, only E6 proteins that are derived from oncogenic HPV types can interact with hDlg, and E6 mutants that can no longer bind hDlg also lose their transforming activity (Kiyono et al., 1997). Moreover, HPV E6 can target hDlg for ubiquitin-mediated degradation (Gardiol et al., 1999; Pim et al., 2000; Kühne et al., 2000), probably by enhancing a normally occurring process, as hDlg appears to be ubiquitinated in cells even in the absence of E6 (Gardiol et al., 1999). Interestingly, hScrib, the human homologue of

Drosophila Scrib, has also recently been shown to be a target for high risk HPV E6-induced degradation (Nakagawa and Huibregtse, 2000). This suggests that the combined targeting of these two cooperating PDZ proteins is essential for viral interference with epithelial cell differentiation. However, there are also important implications for the development of cervical cancer, as misregulation of hDlg and hScrib functions, either by HPV E6 or by cellular mutations, would be expected to affect cell adhesion, polarity and proliferation, thus contributing to the invasiveness of the transformed cells.

Although it is now well established that hDlg is subjected to ubiquitin-mediated degradation by high risk HPV E6, little is known about the regulation of endogenous hDlg protein in normal and transformed epithelial cells. Therefore, in this study we have investigated the role of the proteasome in hDlg regulation in a variety of epithelial cell lines. We show that hDlg is degraded by the proteasome in both the presence and absence of HPV, with the most unstable forms of the protein being hyperphosphorylated. In addition, hDlg becomes intrinsically stabilised upon increased cell contact, but this activity is lost in highly transformed cells. These results demonstrate that hDlg is normally regulated by a complex pattern of events, including phosphorylation and ubiquitination, which in turn are determined by the degree of cell-cell contact, and that loss of this regulation correlates with malignant progression.

MATERIALS AND METHODS

Antibody production

Polyclonal antibodies against Dlg were obtained using the N-terminus of Dlg ,which comprises amino acid residues 1-222 of rat Dlg (Lee et al., 1997), fused to GST. For the first immunisation, 200 μg of purified protein was injected into two New Zealand rabbits in incomplete Freund's adjuvant. In the following six injections 100 μg of protein were injected every 21 days. The specificity of the antisera was tested on western blots of Dlg protein translated in vitro and on protein extracts of HaCaT and CaSKi cells, and compared with the preimmune sera. Finally, no detectable cross reaction was seen against PSD-95 protein (kindly provided by David Bredt), which is the closest PDZ-domain-containing family member to hDlg, thereby confirming the specificity of the antibody.

Cell culture and proteasome inhibition

HPV-16-positive CaSKi, HPV-18-positive HeLa and HPV-negative C33I human cervical carcinoma cell lines, plus the immortalised human skin keratinocytes HaCaT, were all maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum at 37°C and 10% CO_2. Primary baby rat kidney (BRK) cells were obtained from nine-day-old Wistar rats, and the subsequent transformation with HPV-16 E7 plus EJ-ras has been described previously (Massimi et al., 1997). BRK cells were grown under the same conditions as the epithelial tumour-derived cell lines. For proteasome inhibition, growing cells were treated with either N-CBZ-Leu-Leu-Leu-al (Sigma) or N-acetyl-Leu-eu-norleucinal (Sigma) at a final concentration of 50 μM or with 25 μM lactacystin (Calbiochem) for the time indicated, before harvesting for subsequent analysis.

Western blotting

Cells were extracted in a solution of 50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% NP40 and 1% aprotinin. Protein concentrations were determined using the Bio-rad Protein Assay System, and equal amounts (100 μg) were separated on 7.5% PAGE and transferred to nitrocellulose membrane. Endogenous hDlg protein was detected by an anti-Dlg polyclonal antibody and developed with the Amersham ECL System according to the manufacturer's instructions.

Phosphatase assays

Cell lysates were prepared as described above, and 50 μg of protein extract were incubated at 30°C for 30 minutes either with or without 2000 units of λ protein phosphatase (New England Biolabs).

RNA extraction, RT-PCR and Southern blot analysis

Total cellular RNA was isolated from cultured cells with RNAzolB according to the manufacturer's instructions, then DNAse treated and quantified. 5 μg of total RNA were reverse transcribed for 1 hour at 39°C using 200 U of Moloney murine Leukaemia virus reverse transcriptase (Gibco BRL) and either a hDlg-specific antisense primer (5′GTAGAGCTTGGAAGGCTGGAA3′) or a TBP (TATA box binding protein)-specific antisense primer (5′GGTACATGAATTC-CATTACGTCGT3′). 13 cycles of amplification were then performed using either hDlg primers: 5′ATGCCGGTCCGGAAGCAAGAT3′ and 5′GTAGAGCTTGGAAGGCTGGAA3′, or, previously described TBP primers (Massimi et al., 1997): 5′GCTGCGGGATCCAT-GAGGATAAGA3′ and 5′GGTACATGAATTCCATTACGTCGT3′. Amplification conditions were: 1 minute at 95°C, 1 minute at 56°C and 1 minute at 72°C. hDlg (371 bp) and TBP (426 bp) amplificates were then separated on 1% agarose gels and transferred to Hybond-N+ membranes (Amersham). Blots were hybridised with [γ-^{32}P]ATP labelled internal oligonucleotides: hDlg probe 5′CAGACGGCT-TTGAACGATGTA3′ and TBP probe 5′TGGCTCAGAATTCCTA-AATTGTT3′. Quantitation of mRNA expression was done by scanning the Southern blots using a Packard Instant PhosphoImager.

Immunofluorescence assays and confocal microscopy

Cells were washed in PBS, fixed in 3% paraformaldehyde in PBS, incubated for 5 minutes in 0.1% Triton-PBS, stained with α-hDlg monoclonal antibody (2D11 Santa Cruz Biotech., 5 μg/ml) and detected with a FITC-conjugated secondary antibody (Molecular Probes). Images were analysed by confocal laser scanning microscopy with a Zeiss Axiovert 100M microscope attached to a LSM 510 confocal unit.

Anchorage-independence assays

Substrate-independent cell growth was assayed in soft agar. 1×10^5 cells were suspended in growth medium containing 0.5% (w/v) noble agar in 60 mm diameter Petri dishes. Colonies were counted after 10 days.

RESULTS

hDLG is degraded by the proteasome in epithelial cancer cells

We have previously shown that the hDlg protein is targeted for ubiquitin-mediated degradation by the high risk HPV E6 proteins in vitro and, when overexpressed, also in vivo (Gardiol et al., 1999; Pim et al., 2000; Kühne et al., 2000). However, no information was available concerning the levels of hDlg expression in cervical cells containing endogenous E6 protein nor about the cellular pathways that may regulate hDlg in normal and transformed epithelial cells. In order to investigate these aspects, we analysed hDlg protein levels in cells derived from HPV-positive cervical tumours in the presence and absence of proteasome inhibitors. A number of proteasome inhibitors of varying specificity have been described (Rock et al., 1994), therefore we determined the

effects of the inhibitors N-acetyl-Leu-Leu-norleucinal (LL), N-CBZ-Leu-Leu-Leu-al (CBZ) and lactacystin (LC) upon hDlg protein levels in HPV-16-positive CaSKi cells. After two hours of incubation with the inhibitors, equal amounts of whole cell extracts were separated by PAGE and analysed by western blot. hDlg protein was detected with a polyclonal antibody raised against the N-terminal region of Dlg fused to GST. The N terminus comprises Dlg-specific sequences (residues 1-222) and shares no homology with other family members. As can be seen from Fig. 1, the antiserum recognises two major bands, which comigrate with in vitro translated Dlg protein (Gardiol et al., 1999). Interestingly, upon treatment with all three proteasome inhibitors, both forms of hDlg are clearly stabilised, indicating that endogenous hDlg protein is degraded by the proteasome pathway in CaSKi cells. Moreover, additional slower migrating bands also become evident, suggesting that these forms of hDlg are more susceptible to proteasome degradation.

We were interested in comparing the levels of hDlg and its degradation by the proteasome in HPV-16-positive CaSKi cells and HPV-18-positive HeLa cells; therefore cells were incubated with CBZ at a concentration of 50 μM for two and four hours, and hDlg protein levels were then monitored as before. The results are shown in Fig. 2A. Interestingly, the two cell lines differ significantly both in the basal levels of hDlg protein and in the extent of hDlg protein stabilisation achieved upon proteasome inhibition. Indeed, the extracts of HeLa cells appear to contain much less hDlg protein compared to CaSKi cells, and a more prolonged treatment with the proteasome inhibitor CBZ is required for any significant hDlg stabilisation to occur. Coupled with the inability of proteasome inhibitors to be 100% effective, this lower accumulation would suggest a lower expression level in addition to a more efficient degradation of hDlg by the proteasome in HeLa than in CaSKi cells. This observation is consistent with previous data reporting that HPV-18 E6 is a more efficient degrader of hDlg than HPV-16 E6 (Gardiol et al., 1999; Pim et al., 2000). However, incomplete stabilisation of hDlg could also imply a breakdown of hDlg regulation in HeLa cells by another, as yet unknown, mechanism. Interestingly, the higher molecular weight forms of hDlg that appear in CaSKi cells upon proteasome inhibition are not detected in HeLa cells.

Having demonstrated that hDlg protein is regulated by the proteasome pathway in cells expressing high risk HPV E6 proteins, we next examined its regulation in epithelial cells lacking HPV sequences – HaCaT skin keratinocytes and C33I cervical carcinoma cells – as previous evidence had suggested that ubiquitin-mediated degradation of hDlg may occur even in the absence of HPV E6 expression. Fig. 2A shows that HaCaT cells contain relatively high amounts of hDlg and that the protein becomes efficiently stabilised upon proteasome inhibition. As there is no E6 protein in these cells, proteasome-mediated degradation of hDlg must be regulated solely by cellular factors. In contrast, in C33I cells, which harbour much lower levels of hDlg, less hDlg stabilisation is obtained following proteasome inhibition, showing a pattern similar to that in HeLa cells. Therefore it appears that hDlg is normally subject to proteasome degradation in epithelial cells; however, both the protein levels and the extent of degradation are variable between different cell lines. As this could be a consequence of different transcription rates, we next proceeded to analyse the hDlg mRNA expression levels in the different cell lines. We extracted total RNA from CaSKi, HeLa, HaCaT and C33I cells and performed reverse transcription followed by a small number of PCR cycles with specific hDlg primers; Southern blot with an internal probe was then used to detect the amount of product. As a control, TBP mRNA was also

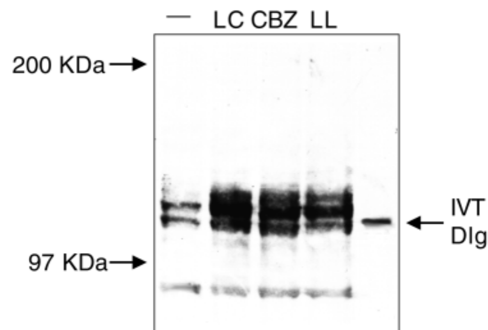

Fig. 1. Regulation of hDlg protein by the proteasome in CaSKi cells. HPV-16-positive CaSKi cervical tumour cells were treated with proteasome inhibitors lactacystin (LC), N-CBZ-Leu-Leu-Leu-al (CBZ) and N-acetyl-Leu-Leu-norleucinal (LL), or with DMSO as control (–) for two hours before harvesting. Equal amounts of protein extracts were then separated by PAGE and analysed by western blot with α-Dlg antiserum. In vitro translated Dlg (IVT Dlg) was included as a positive control and standard molecular weight protein markers (Celbio) are shown.

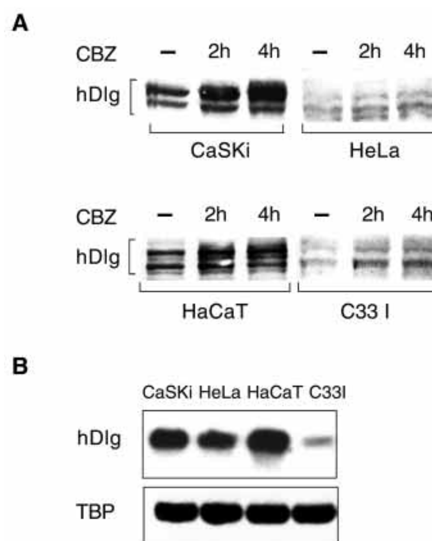

Fig. 2. hDlg degradation by the proteasome in epithelial cancer cells. (A) HPV-16- positive CaSKi, HPV-18-positive HeLa and HPV-negative C33I cervical tumour cells plus HaCaT immortalised skin keratinocytes were treated for either two or four hours with N-CBZ-Leu-Leu-Leu-al (CBZ) proteasome inhibitor before harvesting. Stabilisation of hDlg protein was then assessed by western blot analysis. (B) Comparison of hDlg mRNA levels in CaSKi, HeLa, HaCaT and C33I cells, analysed by RT-PCR amplification (13 cycles) of hDlg mRNA and Southern blot. PhosphoImager scanning of the Southern blot gave the following counts: CaSKi=1335, HeLa=1043, HaCaT=2272, C33I=707. The control was RT-PCR and Southern blot of TBP mRNA.

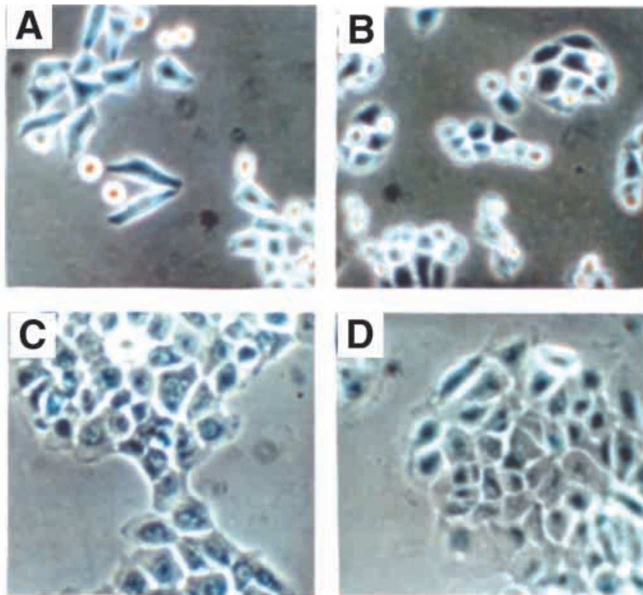

Fig. 3. Comparison of cellular morphologies of the different epithelial cell lines analysed. Direct microscope photographs of cultured cells growing in monolayer are shown. Magnification 60×. (A) HeLa human cervical carcinoma cells (HPV-18 positive). (B) C33-I human cervical carcinoma cells (no HPV).(C) CaSKi human cervical carcinoma cells (HPV-16 positive). (D) HaCaT immortalised human skin keratinocytes (no HPV).

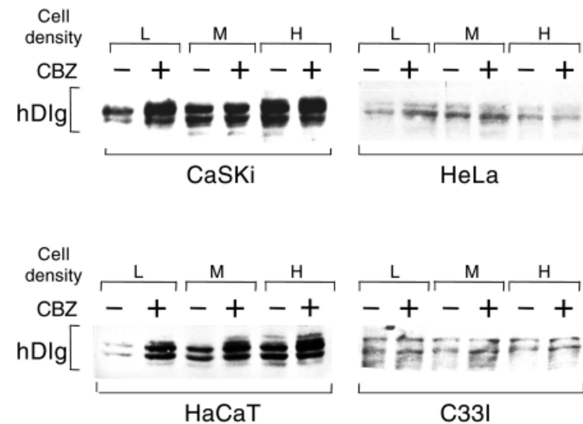

Fig. 4. Density-dependent protein stabilisation of hDlg in epithelial cells. HPV-16-positive CaSKi, HPV-18-positive HeLa, plus HPV-negative HaCaT and C33I cells were grown to 25% (L), 50% (M) or 75% (H) confluence and then either treated (+) or not (–) with the proteasome inhibitor CBZ for four hours prior to cell extraction. Western blot was then performed to analyse hDlg protein levels.

reverse transcribed and amplified from the same samples. Fig. 2B shows that hDlg mRNA levels do indeed vary among different cell lines. In particular, HaCaT cells show the highest mRNA levels, whereas C33I appear to have the weakest expression, and this correlates with the levels of hDlg protein expression. It is also clear from this analysis that in cells with low levels of hDlg (owing to low levels of mRNA) the effects of proteasome inhibition are less apparent. Interestingly, HeLa cells also show lower levels of hDlg transcript compared with CaSKi cells; however this does not fully account for the difference in protein levels, suggesting a higher rate of protein degradation in HeLa cells.

We were intrigued as to the meaning of these differences in the regulation of hDlg between different cell lines, and analysis of the cellular morphology appears to provide an indication. As can be seen from Fig. 3, those cells that have higher levels of hDlg, which increase consistently following proteasome inhibition, such as CaSKi and HaCaT cells, retain relatively differentiated phenotypes and grow in structured, epithelial-like sheets, held together by tight contacts between cells. In contrast, cell lines that contain little hDlg and are less responsive to proteasome inhibition, such as HeLa and C33I cells, show the most undifferentiated phenotypes: these are round cells with disorderly growth that form loose cell-cell contacts. This is reminiscent of the phenotypes described for *Dlg* and *Scrib* mutations in *Drosophila* epithelia (Bilder and Perrimon, 2000), where loss of Dlg function causes both disorganisation of tissue architecture and uncontrolled cell proliferation – crucial steps in the acquisition of an invasive phenotype. To determine if there is a correlation between the degree of invasiveness and the very low levels of hDlg in these epithelial cell lines, we tested their ability to grow in soft agar and the results are shown in Table 1. Interestingly, when cultured in DMEM with 0.5% noble agar, only HeLa and C33I cells were able to form colonies, whereas CaSKi and HaCaT, which retain remarkably higher hDlg levels, were unable to proliferate.

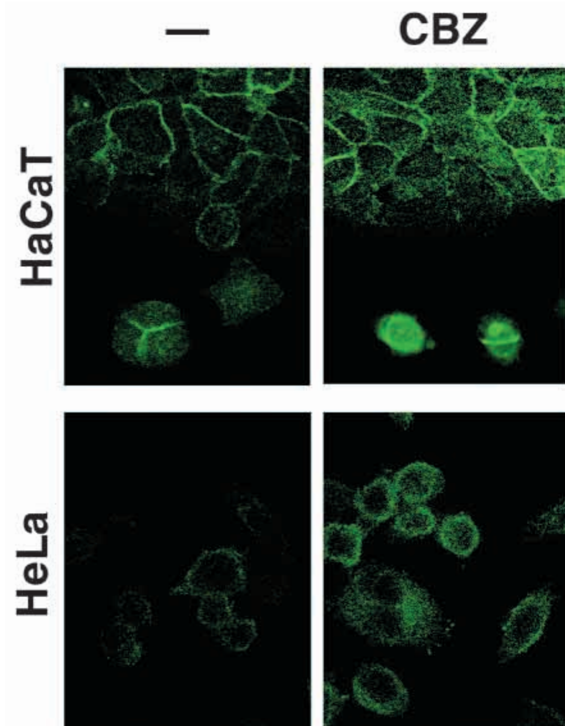

Fig. 5. Localisation of hDlg at cell junctions in differentiated cells inhibits its proteasome degradation. HaCaT skin keratinocytes and HeLa cervical carcinoma cells were either treated or not with CBZ proteasome inhibitor as indicated. hDlg was detected by FITC immunofluorescence and laser confocal microscopy. Representative pictures from 0.8 μm Z-axial slices are shown; settings for scanning were identical.

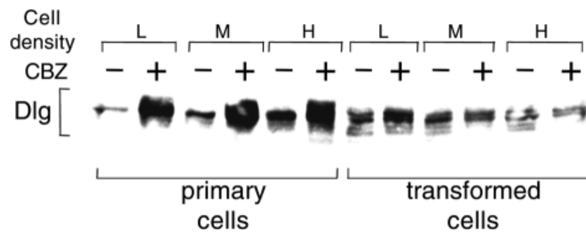

Fig. 6. Consequences of cell transformation on density-dependent stabilisation of Dlg. Primary BRK cells were grown to 25% (L), 50% (M) or 75% (H) confluence and either incubated (+) or not (–) with the proteasome inhibitor CBZ for two hours. The Dlg protein pattern was then analysed by western blot. The same experiment was also performed in parallel on a BRK cell line stably transformed with HPV-16 E7 and EJ-ras.

hDlg protein is stabilised upon increased cell contact

In epithelial cells, E-cadherin-mediated cell-cell adhesion induces the recruitment of hDlg to the lateral plasma membrane and its association with the cortical cytoskeleton at cell junctions (Reuver and Garner, 1998). The finding that cells growing with fewer membrane junctions than a differentiated epithelium also exhibit low levels of hDlg expression, raises the possibility that there is a cellular mechanism for regulating hDlg stability in response to cell-cell contact. To address this issue, we monitored the extent of hDlg degradation in cells growing at different densities to determine the effects of increasing cell contacts on hDlg stability. CaSKi, HeLa, HaCaT and C33I cells were plated at different densities and harvested after two days at approximately 25% (L), 50% (M) and 75% (H) confluence after treatment with either CBZ or DMSO as a control. The cell cycle profiles were also analysed by FACS on both DMSO- and CBZ-treated cells to verify that the cells were growing and that no cell cycle arrest had started even at the highest cell density (data not shown). The results of the western blot are shown in Fig. 4. In the more differentiated CaSKi and HaCaT cells, it is evident that hDlg abundance is lowest at minimum cell density, whereas it increases as cells become more confluent. CBZ treatment demonstrates that these changes in the levels of hDlg are the consequence of the active, proteasome-mediated degradation of hDlg, which is more prominent at low cell density when the cells make few contacts with each other. Indeed, at this stage proteasome inhibition completely restores hDlg protein levels to those reached at the highest cell density. In addition, it is also clear that in CaSKi and HaCaT cells, hDlg becomes intrinsically stabilised as the cells reach confluence and establish a higher number of cell contacts. It is also noteworthy that this density-dependent stabilisation of hDlg is far more efficient in CaSKi and HaCaT cells than in HeLa and C33I cells, which have a less differentiated morphology and, even at high cell density, make very few cell contacts. In both CaSKi and HaCaT cells, stabilisation of hDlg, either by proteasome inhibition or by increasing cell density, invariably results in the protection of previously undetectable slower migrating bands, implying that these forms of the hDlg protein are preferentially degraded. These forms of hDlg were not observed, however, in HeLa nor in C33I cells, where hDlg stabilisation is less efficient.

To further investigate whether hDlg was indeed being degraded by the proteasome in isolated cells, while being stabilised at sites of cell-cell contact, we performed immunofluorescence staining and confocal laser micoscopy using an anti-hDlg monoclonal antibody and a FITC-conjugated secondary antibody. As shown in Fig. 5, HaCaT skin keratinocytes stained for hDlg protein show a weak, diffuse pattern of expression in isolated cells, whereas in groups of cells, hDlg becomes more strongly expressed and is mainly localised at regions of cell contact. However, treatment of cells with the proteasome inhibitor CBZ causes a massive increase in the levels of cytoplasmic hDlg in isolated cells, whereas there is a minimal effect on the amount of hDlg protein being expressed at sites of cell contact. In contrast, analysis of the undifferentiated HeLa cells shows a completely different pattern: hDlg appears to be cytoplasmic in both isolated cells and in groups, and there is no significant membrane localisation even in cells that adhere to each other. Moreover, the slight increase in the levels of hDlg expression obtained following CBZ treatment is not substantially different whether the HeLa cells are isolated or in groups. As expected, analysis of hDlg expression in differentiated CaSKi cells showed a pattern very similar to that of HaCaT cells, whereas cell-junction-dependent stabilisation of hDlg was absent in the undifferentiated C33I cells (data not shown). These results demonstrate that in differentiated epithelial cells, hDlg is stabilised at sites of cell contact. However, any hDlg that is not localised to sites of cell contact is rapidly degraded by the proteasome.

Table 1. Anchorage-independent growth of epithelial cells

Cell type	% of colony formation
HeLa	72±8
CaSKi	0-3
C33I	48±8
HaCaT	0
BRK	0
BRK transformed (HPV-16 E7+EJras)	65±10

For each experiment, 1×10^5 cells were plated in DMEM containing 0.5% noble agar and grown for 10 days. Colonies were then counted and represent the percentage obtained per 500 cells counted – the numbers represent the mean values from three separate assays.

Loss of Dlg expression during malignant transformation

On the basis of the above results it would appear that the ability to regulate hDlg expression is perturbed as cells become less differentiated and display a more transformed phenotype. To directly investigate this, we compared the pattern of Dlg expression in primary rodent epithelial cells and fully transformed cells derived from an identical lineage. Primary BRK cells were prepared from nine-day-old Wistar rats, plated at different densities and treated with the proteasome inhibitor CBZ before analysing Dlg protein by western blot. For comparison, the same experiment was also performed in parallel on a BRK cell line stably transformed with HPV-16 E7 plus EJ-ras only three weeks after the initial transforming event. BRK cells transformed with HPV-16 E7 and ras have been reported to cause tumours in syngeneic rats (Storey et al., 1988), and indeed we have observed that not only our E7/ras

BRK cells are morphologically transformed, with complete loss of cell-cell contacts, but they have also acquired anchorage-independent growth, being able to form colonies in soft agar, whereas their normal counterparts do not (Table 1). As can be seen in Fig. 6, in primary BRK cells Dlg is unstable when the cell density is low, whereas its stability increases as the cells become more confluent. In contrast, the amount of Dlg protein does not increase at a high cell density in the transformed cells and, notably, little stabilisation of Dlg follows proteasome inhibition. These results demonstrate that the ability to stabilise Dlg in response to increased cell contact is indeed lost as cells become fully transformed, suggesting that this loss of Dlg regulation is a key event during the progression of malignancy.

Phosphorylation of hDlg

On western blots of epithelial cells, the anti-Dlg antibody invariably detected several closely spaced bands, which could represent different isoforms of hDlg produced by alternative splicing or postranslationally modified forms of the protein. Cloning of the *hDlg* cDNA has in fact shown that three exons can either be inserted or not, giving rise to at least four potential mRNA isoforms, and Northern blots reveal several *hDlg* transcripts in epithelial cells, consistent with isoform-dependent sequence insertion (Lue et al., 1994). We have shown here that, upon hDlg protein stabilisation in the more differentiated cell lines either as a consequence of proteasome inhibition or of cell contact, the hDlg protein profile on western blots also changes, with the appearance of slower migrating forms of the protein. Therefore, these forms are most probably degraded at a higher rate by the ubiquitin-proteasome pathway in isolated cells while they are stabilised by cell contact, and these may represent 'active' forms of the protein that could have a particular function in establishing cell junctions. It has been reported that CaMKII phosphorylation can affect synaptic localisation of *Drosophila* Dlg (Koh et al., 1999), whereas hDlg interacts with the $p56^{lck}$ tyrosine kinase in human T lymphocytes (Hanada et al., 1997), and it has also been reported to be phosphorylated during mitosis in HeLa cells, possibly by a PDZ-binding kinase (Gaudet et al., 2000). Therefore, in order to determine whether the higher molecular weight species of hDlg were indeed phosphorylated forms, protein extracts of CaSKi and HaCaT cells, either treated or not with CBZ inhibitor, were incubated with λ phosphatase. As shown in the western blot of Fig. 7, in both cell lines the phosphatase treatment dramatically changes the mobility of the higher molecular weight forms of hDlg, demonstrating that they were indeed phosphorylated. These results indicate that hDlg becomes hyper-phosphorylated as a result of increased cell-cell contact and that these phosphorylated forms of hDlg are also more readily degraded by the proteasome under conditions of low cell density.

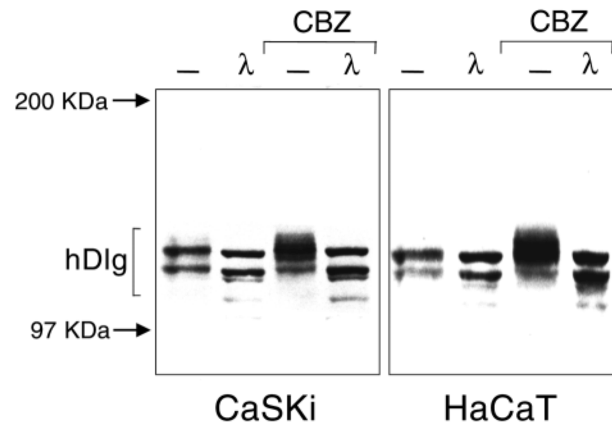

Fig. 7. Accumulation of hyper-phosphorylated hDlg following proteasome inhibition. CaSKi and HaCaT cells were either treated or not as indicated with the proteasome inhibitor CBZ for two hours. After cell lysis, 50 μg of each protein extract were incubated at 30°C for 30 minutes, either with (λ) or without 2000 units of λ protein phosphatase. After separation on SDS-PAGE, the hDlg protein pattern was visualised by western blot analysis. Migration of protein molecular weight markers is indicated.

DISCUSSION

Several proteins involved in cell adhesion are either the product of proto-oncogenes or tumour suppressor genes; the disorganisation of epithelial junctions can lead to defective cell-cell adhesion, loss of cell polarity and unregulated cell proliferation, therefore representing a crucial step in tumorigenesis. The existence of a close connection between regulation of tissue architecture and control of cell proliferation has recently been established by the finding that a small group of *Drosophila* tumour suppressors act in concert to regulate both cell polarity and growth control (Bilder and Perrimon, 2000; Bilder et al., 2000). Mutations in any of these genes cause similar phenotypes, with aberrantly shaped cells, alterations in polarity and overproliferation of epithelial cells. Their products, Dlg, Lgl and Scrib, are membrane-associated proteins that depend on each other for correct localisation and formation of epithelial junctions. Defective cell-cell adhesion could then impair growth control by compromising contact inhibition. Moreover, Dlg is a multidomain protein that can organise the clustering of channels and signalling complexes at cell membranes: mislocalisation of growth factor receptors or signalling molecules, such as Arm or APC, would clearly affect cell proliferation. The modes of action of these onco-suppressors are likely to be conserved in vertebrates: indeed, mammalian Dlg is also present at the lateral membrane in a variety of epithelial cells (Lue et al., 1994; Matsumine et al., 1996). In this tissue, cell-cell adhesion mediated by E-cadherin induces translocation of hDlg from cytoplasmic pools to the plasma membrane (Reuver and Garner, 1998), where it forms complexes with the cytoskeletal protein 4.1 (Lue et al., 1994) and with the APC tumour suppressor protein (Matsumine et al., 1996), thereby inhibiting cell cycle progression (Ishidate et al., 2000). The central role of hDlg, together with hScrib, in maintaining epithelial cells in a differentiated, nonproliferative state is further supported by the fact that both tumour suppressors are targeted for ubiquitin-mediated degradation by the E6 oncoproteins of tumourigenic HPV types (Kiyono et al., 1997; Gardiol et al., 1999; Pim et al., 2000; Nakagawa and Huibregtse, 2000), and expression of high risk E6 has been shown to disrupt epithelial tight junctions (Nakagawa and Huibregtse, 2000). HPVs infect keratinocyte stem cells in the basal layers of the epithelium; however, the replicative phase of the high-risk HPV life cycle is confined to higher levels of the epithelium where keratinocytes are

undergoing terminal differentiation and have ceased cell division (Doorbar et al., 1997). Targeting of both the structural functions of hDlg and its antiproliferative activities may fit into a viral strategy aimed at perturbing cell differentiation and inducing proliferation in order to allow viral replication. However, it can be easily envisaged how losing such restrictions could contribute to the invasiveness of the tumours associated with high-risk HPV types.

In this paper, we show that hDlg is degraded by the proteasome in cell lines derived from cervical tumours containing high-risk HPV. Interestingly, our results also show that HPVs make use of an existing cellular pathway to target hDlg for degradation, as we provide evidence that hDlg protein is intrinsically subject to dynamic regulation via the ubiquitin-proteasome pathway in epithelial cells lacking HPV, such as HaCaT skin keratinocytes and primary rodent epithelial cells. Starting from this finding, the next important step will be to understand how this cellular pathway is modified by the viral oncoproteins and what are the consequences of this for cell fate. Preliminary data indicate that, although in non-synchronised HPV-positive cells the proteasome degradation of hDlg does not appear dissimilar to the HPV-negative cells, cell cycle analyses reveal striking differences, suggesting that HPV-mediated degradation of hDlg takes place during a restricted window of time (P.M., F.M. and L.B., unpublished).

We also show that readily detectable levels of proteasome-regulated hDlg protein are found in those cells that have a more differentiated phenotype. These cells are characterised by the presence of stable junctions that hold the cells together in epithelial structures and regulate their growth upon cell contact. Conversely, low levels of hDlg protein, which is poorly stabilised following proteasome inhibition, were found in those cells that exhibit a more undifferentiated phenotype, make very loose cell contacts and grow in a disorderly fashion without contact inhibition. Thus, loss of hDlg expression correlates with a more undifferentiated phenotype, and this would appear to occur, in part, at the level of mRNA expression. Moreover, the levels of hDlg protein are regulated by the degree of cell contact. Thus, in isolated cells hDlg is continually degraded by the proteasome pathway; however, once contacts with neighbouring cells are established, the hDlg protein, which localises at membrane junctions, becomes stabilised. Again, stabilisation is only seen in the more differentiated cell types and appears to be independent of the presence of HPV sequences. Interestingly, analysis of primary BRK cells also demonstrates a very similar pattern of Dlg regulation; however, once those cells become fully transformed, the ability to regulate Dlg is lost, together with the capacity to form cell junctions, and the protein fails to accumulate even when the cells reach high density. These results suggest that the loss of hDlg expression, and in particular, loss of the ability to upregulate it upon cell contact, is a vital step during malignant progression. Indeed, we have observed that in all cells that have impaired Dlg regulation there is a parallel acquisition of an invasive phenotype and of a capacity to grow in an anchorage-independent manner. Although the cellular roles of hDlg are still largely unknown, there is growing evidence that it may have tumour suppressive functions in regulating cell polarity and proliferation. It would therefore be of great interest to determine if loss of hDlg expression is a common feature in all highly malignant tumours.

Not all forms of the hDlg protein are degraded with equal efficiency by the proteasome. Some of them are in fact eliminated more dramatically, being stabilised only after treating the cells with proteasome inhibitors or by cell contact. This would also imply their direct involvement in the molecular organisation of the epithelial junctions. Previous reports have suggested that different isoforms of hDlg might exhibit exclusive binding properties, thus being able to perform specialised functions in the cell. The binding site for the cytoskeletal protein 4.1 has been mapped to an alternatively spliced domain of hDlg (Marfatia et al., 1996), which may therefore be involved in its subcellular targeting. Another alternatively spliced domain of hDlg encodes a potential SH3-binding site in the N-terminus, a region that mediates its selective recruitment to sites of cell contact (Wu et al., 1998). Using phosphatase experiments, we show that the most unstable forms of hDlg, which become stabilised upon increased cell density, are phosphorylated. This finding further supports the idea of different hDlg pools performing specific roles that need to be tightly regulated in the cell. Post-translational modifications have been shown to regulate the membrane targeting of several Dlg family members: palmitoylation controls association of PSD-95 with the plasma membrane (Topinka and Bredt, 1998; El-Husseini et al., 2000), whereas phosphorylation of insect Dlg by CaMKII regulates its anchoring to the synaptic complex (Koh et al., 1999), and phosphorylation of the MAGUK ZO-1 has been proposed to play a role in the assembly of tight junctions (Kurihara et al., 1995). Phosphorylation could also be part of a signalling cascade that regulates proteasome degradation of hDlg. β-catenin, a protein involved in both cell-cell adhesion and growth factor signal transduction, is part of such a cascade. Upon phosphorylation, β-catenin is targeted for proteasome degradation (Orford et al., 1997) and, interestingly, also forms a complex with hDlg and APC (Matsumine et al., 1996).

In this paper we have begun to elucidate the complex and dynamic pattern of hDlg protein regulation. This is reminiscent of β-catenin regulation, and it is therefore tempting to hypothesise that hDlg also functions in cell signalling, in addition to its roles in the organisation of membrane junctions. hDlg belongs to a family of proteins called membrane-associated guanylate kinase homologues (MAGUKs) which have indeed been shown to be involved in cell signalling in *Caenorhabditis elegans* vulva precursor cells (Hoskins et al., 1995), and several lines of evidence suggest that hDlg may also transduce growth inhibitory signals. It has long been known that some mutations in *Drosophila Dlg* cause neoplastic growth of epithelial cells (Woods and Bryant, 1989) without affecting the structural functions of the protein (Woods et al., 1996), and although now its role in suppressing cell proliferation is well established, the underlying molecular mechanisms are only starting to be investigated. For example, overexpression of hDlg is able to block cell cycle progression from the G_0/G_1 to S phase (Ishidate et al., 2000), probably by means of its interaction with APC (Matsumine et al., 1996), as mutant APC lacking a hDlg-binding motif exhibits weaker antiproliferative activity (Ishidate et al., 2000). Phosphorylation and ubiquitination of hDlg, regulated by cell contact, may well modulate interactions of hDlg with its protein partners, thereby affecting downstream pathways.

In conclusion, our findings support the notion that hDlg

plays an active role in the molecular organisation of epithelial junctions and in the maintenance of a differentiated epithelial architecture. Moreover, we provide evidence that, in epithelial cells, hDlg is subjected to a complex pattern of regulation through both phosphorylation and ubiquitination. These events can modulate hDlg stability in a dynamic fashion, depending upon the extent of cell-cell contact, and they may also affect additional functions of hDlg that regulate cell proliferation. Work is in progress to investigate in further detail the molecular mechanisms of hDlg regulation and their consequences, as well as the events that correlate their misregulation with the undifferentiated and invasive phenotype.

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