ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Novel cellular pathways inactivated by the HPV E6 transforming protein.

Thesis submitted for the Degree of Doctor Philosophiae

Candidate:
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Supervisor:
Dr. Lawrence M. Banks

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Questa Tesi e' dedicata a Romeo
che, leggendo "Le Scienze" fra un lavoro di cucina e l'altro,
mi ricorda ogni giorno cosa sia la passione per la scienza.

Alla (sempre piu') grande famiglia dell'Osteria
vanno il mio affetto e la mia gratitudine
per avermi aiutata, sostenuta e sfamata
in questo come in tutti i momenti importanti della mia vita.

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SUMMARY

The human papillomavirus (HPV) E6 is one of three oncoproteins encoded by the virus. It has long been recognised as a potent oncogene and is intimately associated with the events that result in the malignant conversion of virally infected cells. The aim of this thesis is to investigate how E6 impinges upon diverse cellular pathways, whose inactivation appears central to tumour progression.

Firstly, the activation of the p53 tumour suppressor was examined in HPV-positive cancer cells, in conditions where E6-mediated degradation was blocked, with the aim of studying whether E6 interferes with the functions of p53 independently of targeting it for degradation. It was found that inhibition of proteasome activity in HPV-containing tumour cells does not always result in activation of p53, moreover, in E6 expressing cells the nuclear localisation of p53 in response to DNA damage is also blocked, consistent with previous observations which indicate that degradation of p53 is not essential for E6 mediated inhibition of p53 functions.

Several PDZ-containing proteins have recently been shown to be crucial targets for the tumour-promoting activities of high-risk E6. Therefore, the activity of HPV E6 towards the multi-PDZ tight junction protein MUPP-1 was analysed, demonstrating that high-risk HPV E6 binds MUPP1 through its C-terminal PDZ-binding motif and targets it for ubiquitin-mediated degradation.

Finally, since high-risk HPV E6 has been demonstrated to target the putative tumour suppressor protein Dlg for degradation, the cellular mechanisms which regulate the stability of this protein were studied in both HPV-positive and negative epithelial cells. This study highlighted a complex pattern of hDlg regulation through both phosphorylation and ubiquitin-mediated degradation in response to cell-cell contact. The use of models of tumour progression *in vitro* also demonstrated that loss of this regulation is likely to represent a significant step in the development of malignancy. These results have important implications for any potential therapies which might aim to block the interactions of HPV E6 with some of its cellular targets.

INTRODUCTION

Virus-induced carcinogenesis involves a remarkable variety of mechanisms, either direct or indirect. Most frequently, viruses contribute directly to tumour development, and the expression of viral oncogenes is required for the establishment and maintenance of the transformed phenotype. Viral oncoproteins can modify a set of cellular proteins, either changing their expression pattern, altering or completely inactivating their function. As a consequence, intracellular signalling cascades that stimulate cell proliferation or block senescence pathways are affected, leading to outgrowth and immortalisation of target cells. Malignant progression also involves the inactivation of intercellular regulatory mechanisms, with the consequence of rendering the transformed cells unresponsive to growth inhibitory signals from neighbouring cells and tissues. Finally, disruption of cell polarity control and loss of anchorage dependence contribute to the metastatic potential of tumour cells. Frequently viral infection can affect tumour development also indirectly, for instance by inducing immunosuppression or by preventing apoptosis of target cells, thereby allowing the survival of cells harbouring activated oncogenes or mutated tumour suppressor genes. Clearly host cell transformation is not the ultimate aim of a viral infection, while it represents a rare and unfortunate event also from the viral point of view. Infection by human Papillomaviruses, Hepatitis B virus, Epstein-Barr virus, human Herpesvirus and human T cell leukemia-lymphoma virus have been consistently linked to specific malignancies, and together account for 20% of the global cancer burden (zur Hausen, 2001). However, none of these infections *per se* is sufficient to induce cancer, whose development requires the cooperative effect of environmental carcinogens.

The study of oncogenic DNA viruses is extremely important, because it provides us with powerful tools to address the mechanisms of tumorigenesis, exemplifying the multifactorial and multistep nature of this process. An equally important goal is the development of effective therapeutic strategies against specific virus-induced cancers, many of which represent extremely serious problems for public health.

Papillomaviruses.

Papillomaviruses (PVs) are small double stranded DNA viruses, which cause a spectrum of epithelial proliferative lesions ranging from warts to cancer. These viruses have two special properties which define their biology: exquisite species specificity (human PVs only infect humans etc.) and tissue tropism, i.e. the productive viral cycle is obtained only during keratinocyte differentiation. At present there is not an efficient and widely available tissue culture system that supports a complete infectious cycle, and the viruses are not classified as serotypes but as genotypes (Stanley, 2001). In humans, probably because there has been an intense search for viral genomes in many tissues, there is a remarkable plurality of PV types with more than 130 genotypes identified (de Villiers, 1997), however this number can further increase including those HPV types only partially characterised by PCR amplified fragments (de Villiers, 1999). Within a species the individual viruses show a predilection for either cutaneous or mucosal sites, and within the groups of mucosal and cutaneous viruses they can be separated into high- or low-risk types, depending upon their oncogenic potential. The DNA of more than 80 genotypes has now been sequenced and overall there is a high degree of conservation of genomic organisation (Figure 1). The HPV genome can be divided into 3 domains: a non-coding upstream regulatory region (URR) of approximately 1 Kb, an early region with open reading frames (ORFs) E6, E7, E1, E2, E4 and E5, and a late region encoding the two capsid proteins L1 and L2. The functions of the ORFs are described in Table 1.

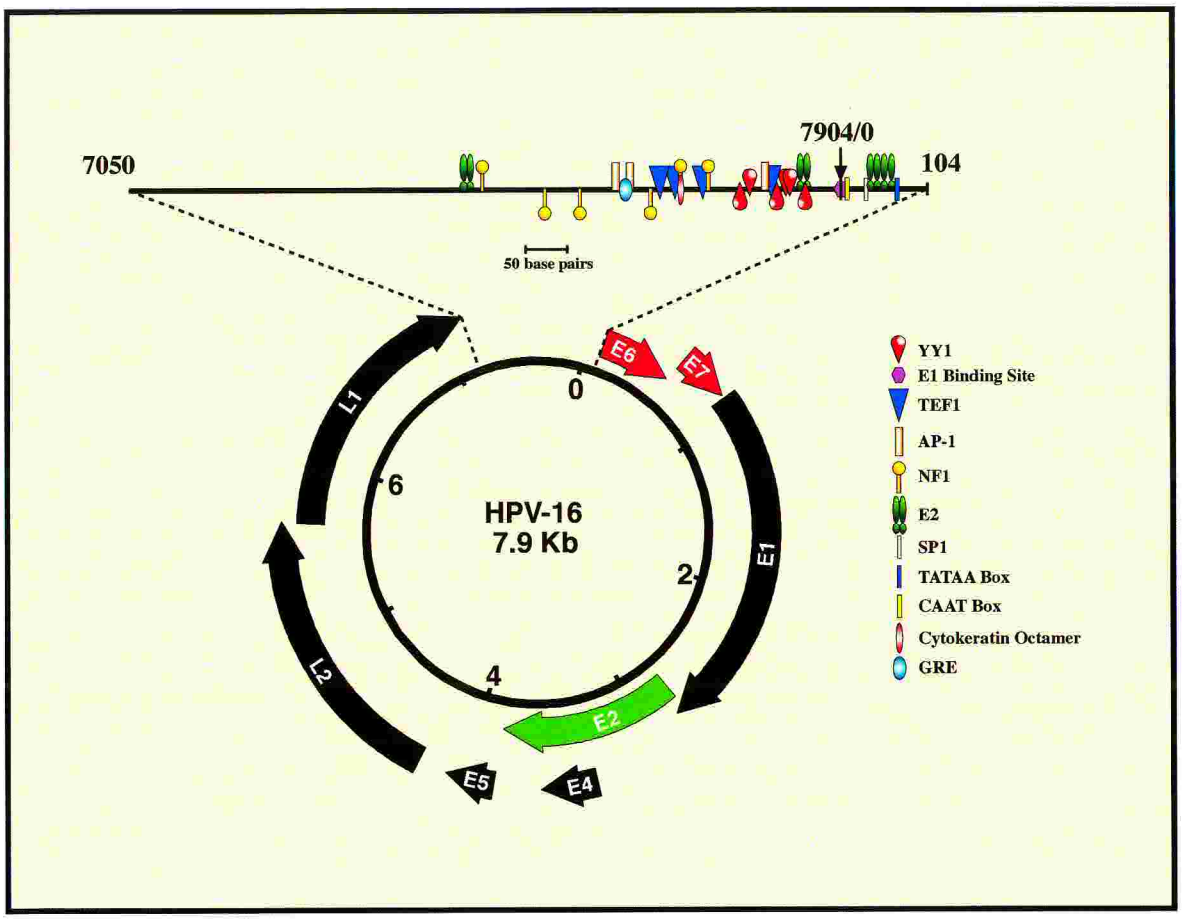


Figure 1. Genome organisation of human papillomavirus type 16.

Table 1

ORF	Functions
E1	Viral DNA replication, only viral enzyme (helicase, ATPase)
E2	Viral DNA replication and transcription. Forms a complex with E1 which can then bind with higher affinity to the ORI; transcriptional activator and repressor of the viral early promoter
E6	Transactivation, suppression of apoptosis, mitogenic factor, disrupts cell junctions
E7	Mitogenic factor: binds pRB, induces unscheduled DNA synthesis in differentiating keratinocytes
E5	Mitogenic factor; interacts with growth factor receptors, causes accumulation of nondegraded EGF-receptor
E4	Binds keratins and induces cytoskeletal changes; interacts with DEAD Box protein: putative role in late protein synthesis
L1	Major capsid protein
L2	Minor capsid protein

Infectious cycle of the papillomaviruses.

The papillomavirus life cycle is absolutely dependent upon the differentiation program of the keratinocyte (Stubenrauch and Laimins, 1999; Stanley, 2001, for review). Only keratinocytes or cells with the potential for squamous maturation (such as the reserve cell of the squamocolumnar junction in the cervix) are permissive for viral gene expression and only in terminally differentiated keratinocytes are viral capsid proteins synthesized and viral particles assembled.

The receptor for HPV entry into epithelial cells has not been functionally identified, although the integrin α6-β4 protein complex has been put forth as a candidate receptor (Evander et al., 1997). This integrin is present on epithelial stem cells (Li et

al., 1998) and is expressed during wound-healing, which makes it a logical candidate, however no functional studies have yet shown it to mediate HPV entry. Furthermore, HPV virions also bind to heparin, a ubiquitous polysaccharide which may provide the initial attachment event, that is then followed by association with a receptor complex and internalisation (Joyce et al., 1999). Presently it is not clear if the cellular proteins mediating viral entry contribute also to cell type specificity or if other molecules such as transcription factors play the major regulatory roles.

The productive life cycle of all PVs is coupled to cell differentiation (Figure 2). Initial infection occurs into epithelial stem cells located in the lower layers of the stratified epithelium: these cells divide producing daughter cells, which withdraw from the cell cycle, migrate away from the basal layer and become committed to differentiation. Following entry into the cell, the viral genome is established as extrachromosomal element in the nucleus and copy number is increased to 50-100 copies per cell. As infected cells divide, viral DNA is distributed equally among daughter cells: while one migrates away and initiates a differentiation program, the other continues to divide thereby providing a reservoir of viral DNA. Since viral production is restricted to differentiating cells, the infection can persist in the basal layers for several years (Stubenrauch and Laimins, 1999).

Following infection, the first viral genes to be expressed are the replication proteins, E1 and E2. E2 binds with high affinity to origin sequences in the URR and to E1, thereby allowing formation of a multimeric complex that facilitates stable binding of E1 to the viral origin of replication (Sedman and Stenlund, 1995; Sanders and Stenlund, 1998). E1 has helicase and ATPase activities (Yang et al., 1993; Hughes and Romanos, 1993) and also recruits cellular replication enzymes to the viral ori (Park P. et al., 1994; Bonne-Andrea et al., 1995; Masterson et al., 1998). Transient replication of HPV genomes only requires E1 and E2, but stable maintenance in normal keratinocytes also requires E6 and E7 expression (Del Vecchio et al., 1992; Stubenrauch et al., 1998; Thomas J. et al., 1999). This suggests that the E6 and E7 proteins must modify the cellular environment to permit maintenance of the extrachromosomal elements and prevent their rapid removal. In normal epithelia,

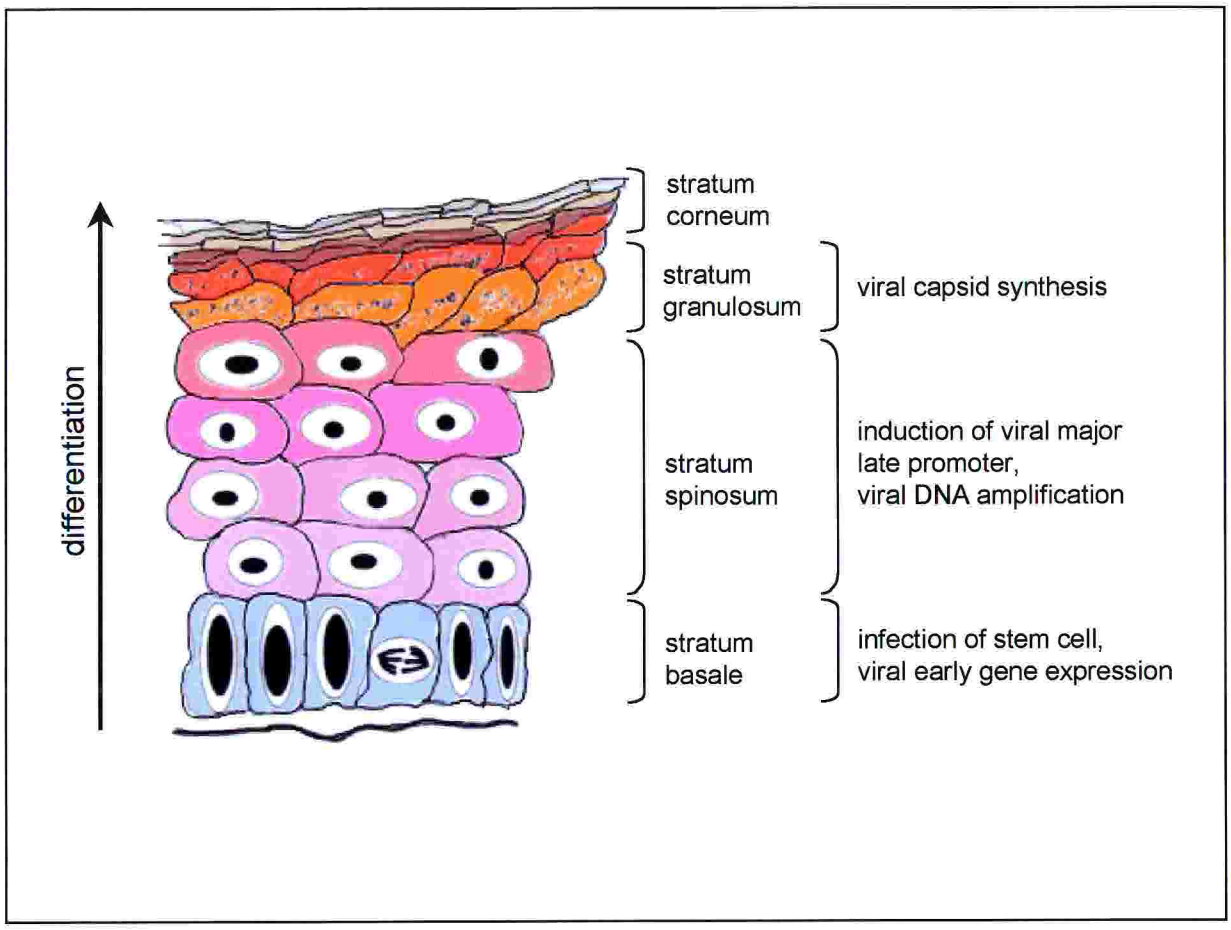


Figure 2. Differentiation dependent functions in HPV-infected epithelial cells.

differentiation is coupled to cell cycle exit. Since HPVs rely on cellular enzymes to replicate their genomes, one major consequence of HPV infection is blockage of cell cycle exit. HPV-infected cells undergo a last S-phase in differentiated suprabasal cells to replicate HPV genomes to high levels prior to packaging into newly assembled capsids (Laimins, 1996). However, the replicative phase of high-risk HPV infection is confined to more differentiated cells, that have already exited cell cycle and are normally non-permissive for DNA synthesis (Doorbar et al., 1997). Thus, the high-risk E6 and E7 proteins act as viral replication competence factors: E7 targets a number of cell cycle regulatory proteins, thereby stimulating G1/S transition and DNA synthesis. The host cell's normal response to such unscheduled induction of proliferation would be, however, to trigger apoptosis and/or growth arrest. To overcome these obstacles, the high-risk E6 protein targets a variety of cellular proteins involved in regulating these mechanisms, as well as terminal differentiation and antiviral defence (see below).

Expression of HPV late genes is regulated by epithelial differentiation, however our knowledge of how this process occurs is limited. In one model, the key event to the activation of late viral functions is induction of the late promoter by differentiation-specific transcription factors. In a second model, the activation of viral replication is an initial event that then leads to titration of transcription factors, which repress late expression in basal cells. Upon differentiation, transcription of E1 and E2 switches to a late promoter, which is not repressed by E2 (Klumpp and Laimins, 1999). This results in increased levels of E1 and E2 leading to amplification of the viral genomes. The major late transcripts encode an E1-E4 fusion protein (Nasseri et al., 1987), which is thought to facilitate capsid release by perturbing the organisation of the cytokeratin matrix (Doorbar et al., 1991), as well as E5 whose action is also related to mitogenic stimulation (see below). The capsid proteins, L1 and L2, are translated from messengers which also encode E1-E4 upstream (Doorbar et al., 1990). Interestingly, the HPV-16 E1-E4 protein interacts with a member of the DEAD box protein family of RNA helicases. This protein complex binds RNA, including the major late transcript E1E4.L1 and is able to shuttle between nucleus and cytoplasm, indicating a possible

role in capsid protein synthesis by regulating the stability and/or the translation of late viral transcripts (Doorbar et al., 2000). It has been recently proposed that restricting the translation of the late viral transcripts to differentiated keratinocytes is achieved by differential codon usage, which matches the specific composition of the tRNA population in these cells (Zhou et al., 1999). Viral assembly occurs as cells reach the epithelial surface and die (Stoler et al., 1990), allowing for release of new infectious virions: since HPVs do not fully lysate their host cells, virus particles are deposited within the epithelial squames that are constantly shed.

Association between HPV infection and cancer.

The continuous proliferation of epithelial cells triggered by an HPV infection results in the characteristic benign wart or papilloma from which the virus name is derived. On rare occasions however, the infectious life cycle can be interrupted, and the viral DNA integrates at random sites within the cellular genome. Its integrity is often disrupted, resulting in deregulated expression of the E6 and E7 ORFs. This is tightly correlated with the initiation of processes leading to immortalisation and ultimately full transformation of the host cells. Lesions can progress through mild or severe dysplasia, in some cases becoming invasive and metastatic.

Epidemiological studies have established HPVs as the causative agents of a number of important human cancers, the best characterised of these being cervical cancer, where the virus is found in over 99% of cases. This cancer is the third major cause of cancer-related death in women worldwide, with over 370,000 new cases per annum (Parkin et al., 1999). HPVs are also associated with the development of cutaneous squamous cell carcinoma (SCC), particularly in immuno-compromised individuals, where between 34-50% of patients surviving 15 years after renal transplantation develop skin cancer (London, et al., 1995; Benton and Arends, 1996). Only a small subset of the numerous HPV types known are associated with the development of cancer (de Villiers, 1994) and are accordingly classified as either high-risk or low-risk types. The

major high-risk types associated with cervical cancer are HPV-16, 18, 31 and 33 (zur Hausen and Schneider, 1987; zur Hausen, 1991). Other viral types such as HPV-6 and HPV-11 are regularly found associated with benign lesions or occasionally with low-grade dysplasias of the cervix, and therefore defined as low-risk types.

The low-risk and the high-risk HPVs are both highly successful viruses that infect similar tissues, and it is therefore logical to ask which biological activities of the high-risk types are responsible for their much higher transforming potential. The structures and presumably the functions of their encoded transforming proteins are quite similar. However, there are many biological activities, which are not shared between the low-risk and the high-risk E6 and E7 proteins (see below). In fact, important aspects of their life cycles are different: as discussed above, there is a spatial and temporal difference between the low- and high-risk HPVs in their sites of DNA replication within the differentiating epithelium (Doorbar et al., 1997). The replication of the low-risk HPVs is generally restricted to the lower levels of the stratified epithelium, where the keratinocytes are still undergoing cell division: these viruses perturb the normal differentiation pathways of the infected cells causing highly visible proliferations. In contrast, the high-risk HPVs replicate their genomes in the higher levels of the epithelium where the keratinocytes are undergoing terminal differentiation; these infections lead to less perturbation of the normal differentiation pattern of the host cell, thus producing smaller, less obvious lesions. At the same time however, the high-risk E6 and E7 functions need to be more powerful in order to push their host cells to restart proliferation in a more unnatural environment than their low-risk counterparts. It can also be speculated that for the same reason high-risk specific activities have evolved to maximise viral replication and persistence for this class of viruses. On the other hand, it is not difficult to envisage how deregulated expression of these viral proteins can drive the cell towards transformation.

The functions of the HPV oncogenes.

HPV E5

The E5 proteins are approximately 10 kDa in size, highly hydrophobic, type II transmembrane proteins that exist as homodimers and localise to the membranes of the endoplasmic reticulum, Golgi apparatus and nucleus of transformed cells (Conrad et al., 1993). Their function is related to cell proliferation and mitogenesis, and although devoid of intrinsic enzymatic activities, they act by modulating the function of cellular membrane proteins that regulate cell growth (DiMaio and Mattoon, 2001, for review). Due to the weak intrinsic transforming activity of the HPV E5 proteins (Leptak et al., 1991; Pim et al., 1992), most of the early work was done on the BPV equivalent, which exhibits a far higher transforming potential. BPV E5 can induce morphologic and tumourigenic transformation of rodent and human fibroblasts (Schlegel et al., 1986; Surti et al., 1998).

Several lines of evidence suggest that tyrosine kinase signalling plays an important role in transformation by E5. BPV E5 protein has been shown to cause the ligand-independent activation of the PDGF β receptor, but not of the closely related PDGF α receptor (Petti et al., 1991; Petti and DiMaio, 1992). E5 acts by mimicking the action of the natural ligand PDGF: it binds to the receptor, although not in the PDGF-binding site, and induces its dimerisation and trans-phosphorylation, which initiates the signalling cascade (Lai et al., 1998). Moreover, it can induce downregulation of the receptor, similarly to the effect produced by PDGF (Nilson et al., 1995; Petti and Ray, 2000). Activation of PDGFβ–R is required for cell transformation by BPV E5 (Drummond-Barbosa et al., 1995; Goldstein et al., 1994) and provides an anti-apoptotic signal in the face of growth factor deprivation.

On the other hand the EGF receptor, rather than the PDGF β receptor, appears to be involved in transformation by HPV E5. This difference is consistent with the tissue tropism of the HPVs, which infect exclusively epithelial cells, a cell type rich in EGF receptors but thought to be devoid in PDGF β receptors. In contrast, BPV also infect

mesenchimal cells that abundantly express PDGF β receptor. Consistently, HPV-16 E5 can induce anchorage-independent growth in cells lacking EGFR only when the viral gene is co-expressed with the EGFR gene, and colony formation is enhanced by treatment with EGF but not with PDGF (Pim et al., 1992). In contrast to the ligand-independent activation of the PDGF β receptor caused by BPV E5, HPV E5 primarily affects the metabolism of the EGF receptor. It reduces the ligand-induced degradation of the activated EGFR in endosomes and increases its recycling to the cell surface (Crusius et al., 1997; Straight et al., 1993, 1995), thereby rendering the cells more sensitive to EGF. This appears to be mediated by a reduction in the acidification of the endosomes involved in receptor processing (Straight et al., 1995), most likely through the ability of the E5 proteins to complex with the 16 kDa component of the vacuolar H^+ATPase (Goldstein et al., 1991; Conrad et al., 1993). Gap junction-mediated cell-cell communication is also inhibited in keratinocytes expressing HPV-16 E5 (Oelze et al., 1995), possibly rendering the transformed cells insensitive to growth inhibitory signals emanating from neighbouring cells. This may also be a consequence of E5 association with the vacuolar ATPase, which appears to be a component of gap junctions.

However, interactions with the EGFR do not account for all the effects of the HPV E5 protein on cellular signal transduction pathways (Crusius et al., 1999). Keratinocytes expressing the HPV-16 E5 protein display increased DNA synthesis and serum-free proliferation in response to endothelin-1, suggesting that the viral protein also enhances signalling via the G protein-coupled endothelin receptor (Venuti et al., 1998), however this effect does not appear to involve increased levels of the receptor.

The consequences of growth factor receptor activation by E5 include increased activation of MAPK (Gu and Matlashewski, 1995; Crusius et al., 1997) and a concomitant upregulation of the early response genes c-jun and c-fos (Bouvard et al., 1994). There is also evidence that E5 may activate the PKC pathway, also giving rise to an increase in early gene expression (Crusius et al., 1997). Therefore, E5 can be viewed as activating the very early G1 phase of the cell cycle in infected cells. In terms of viral replication, this will most likely prime the cell for the stimulatory effects of E7 on

DNA replication (see below). An additional consequence of c-jun and c-fos upregulation is the activation of viral gene expression from the URR regulatory region, that contains recognition sites for a variety of transcription factors, including AP-1 (Chan et al., 1990; Bouvard et al., 1994). E5 might therefore increase the expression of the other early genes E6 and E7 and thus contribute to the early steps of immortalisation. During the development of cervical tumours, deletions frequently occur that encompass the HPV E5 gene, and it is therefore generally assumed that E5 has no role in the later stages of malignancy. However it is worth noting that, although E5 is frequently deleted, the EGFR genes are often amplified in cervical tumours, possibly compensating for E5 loss (Bauknecht et al., 1989; Köhler et al., 1989).

HPV E7

E7 is one of only two viral proteins that remain expressed in cervical tumours and derived cell lines (Smotkin and Wettstein, 1986; Seedorf et al., 1987), being required for maintenance of the transformed phenotype (von Knebel Doeberitz et al., 1988; Alvarez-Salas et al., 1998; Wang-Johanning et al., 1998). HPV E7 are small, acidic phosphoproteins composed of approximately 100 amino acid residues (Münger et al., 2001, for review). The amino terminus shows sequence homology to the conserved regions CR1 and CR2 of Adenovirus E1A and SV40 TAg, which is also reflected by some similarity in the mechanisms used by these DNA tumour virus proteins to alter cell proliferation (Phelps et al., 1988; Phelps et al., 1991). The carboxyl terminal half of E7 comprises a zinc finger and, intriguingly, the HPV E6 proteins contain two tandem copies of this domain, leading to the speculation that E6 and E7 may have evolved through duplication of an ancestral viral gene.

As already discussed, the productive life cycle of papillomaviruses occurs in differentiating epithelial cells which, however, having stopped DNA synthesis, are not inherently capable of supporting viral replication. Several studies have shown that the ultimate effect of E7 expression is to subvert the tight link coupling cellular

differentiation with the termination of proliferation in normal epithelium, hence retaining the differentiating keratinocytes in a DNA replication competent state (Cheng et al., 1995; Woodworth et al., 1992). This is achieved through a variety of biological activities, which are responsible for the transforming potential of E7, once its expression becomes uncoupled from the viral life cycle.

The best studied cellular targets of E7 are the pocket proteins pRB, p107 and p130, which are major regulators of the cell cycle, responsible for modulating the expression of genes required for cell cycle progression. Hyperphosphorylation of pRB by cyclin-dependent kinases (CDKs) and subsequent release of free E2F transcription factors is required for S phase entry (Nevins et al., 1992, for review). The E7 proteins from the high-risk group of HPVs interact with pRB more strongly than those from low-risk HPVs (Münger et al., 1989a), and this may account in part for differences in their respective transforming activities. E7 binds to the "pocket domain" of pRB, p107 and p130, via an LXCXE motif lying in the CD2 region (Münger et al., 1989a; Dyson et al., 1992). This interaction is however not sufficient for both release of free E2F and inhibition of pRB DNA binding, and sequences within the C-terminal half of E7 are also required (Huang et al., 1993; Patrick et al., 1994). In fact, E2F does not contain an LXCXE motif and binds to a different region of pRB than HPV E7 (Dick et al., 2000; Huang et al., 1993; Wu E. et al., 1993). Recent studies have shown that binding to high-risk HPV E7 results in the destabilisation of pRB and related pocket proteins (Boyer et al., 1996; Jones and Münger, 1997). A potential mechanism is suggested by the discovery that E7 interacts with the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi, 1997), thus increasing its ATPase activity. Since E7 is expressed at low levels, this represents an extremely efficient strategy to inactivate more abundant cellular proteins, and also allows the abrogation of E2F-independent activities of the pocket proteins, as those related to senescence and differentiation (Sellers et al., 1998). Consistent with this notion, it was shown that expression of E7 and its ability to subvert cellular targets such as pRB are indispensable for HPV replication and the full viral life cycle (Flores et al., 2000; Thomas J. et al., 1999). The pocket domain of pRB, p107 and p130, is also required for binding to a range of

cellular proteins containing an LXCXE motif, among which are cyclins D1 and D2, and histone deacetylases (HDAC) 1 and 2. During the G1 phase of the cell cycle, pRB actively represses genes required for progression to S phase by recruiting HDACs at their promoters (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). HPV-16 E7 has been shown to be able of disrupting these complexes (Brehm et al., 1998). Other studies have indicated that both p107 and p130 are also able to recruit HDACs, and since Adenovirus E1A has been shown to disrupt these interactions (Ferreira et al., 1998), it seems probable that E7 may also do so.

The ability of E7 to abrogate the activities of cyclin-dependent kinase inhibitors such as $p21^{CIP1}$ (Funk et al., 1997; Jones et al., 1997a) and $p27^{KIP1}$ (Zerfass-Thome et al., 1996) is also important for the viral requirement of uncoupling the processes of cellular differentiation and proliferation. Several studies have shown that $p21^{CIP1}$ plays an important role in coupling cell cycle arrest and differentiation in keratinocytes (Alani et al., 1998; Di Cunto et al., 1998; Missero et al., 1996). The steady-state levels of $p21^{CIP1}$ increase during cellular differentiation independently of p53, resulting in inactivation of cdk2 activity and growth arrest.

E7 has been shown to sequentially activate the cyclin E and cyclin A promoters (Zerfass et al., 1995), and to upregulate the cellular levels of Cdc25A phosphatase (Katich et al., 2001), which activates cyclin E/cdk2 and cyclin A/cdk2 complexes. Moreover, E7 has been found to become part of two cyclin-containing complexes: the S-phase-specific E2F/cyclin A/p107/cdk2 complex (Arroyo et al., 1993), and the G1-specific E2F/cyclin E/p107/cdk2 complex (McIntyre et al., 1996).

E7 has also been found to activate transcription independently of pRB binding (Zwerschke et al., 1996). This activity is explained by the finding that E7 can interact with the core component of the TFIID transcription factor complex, the TATA-box binding protein (TBP) (Massimi et al., 1996), and with the TBP-associated factor TAFII10 (Mazzarelli et al., 1995). In addition to associating with components of the basal transcription machinery, E7 has been shown to interact with members of the AP-1 family of transcription factors (Antinore et al., 1996).

HPV-16 E7 has been found to bind to and inactivate the insulin-like growth factor binding protein 3 (IGF-BP3) (Mannhardt et al., 2000), which is the product of a p53-inducible gene, and has cytostatic and proapoptotic effects (see below). Experiments using proteasome inhibitors suggest that E7 induces the degradation of IGF-BP3 at the 26S proteasome. Moreover, the high-risk HPV E7 proteins have been shown to abrogate the cytostatic activities of cytokines as TGF–β (Pietenpol et al., 1990b), TNF α (Basile et al., 2001), and IFNs (Barnard et al., 2000; Park et al, 2000).

Abnormal proliferative signals such as oncogene activation and loss of functional RB are associated with the induction of a p53-mediated cellular response. In fact, expression of HPV-16 E7 in normal diploid fibroblasts (NDFs) gives rise to an increase in the levels of p53 (Jones et al., 1997b), and it appears to do so by inhibiting the interaction between p53 and Mdm2 (see below) (Seavey et al., 1999). This effect is, at least in part, independent of ARF, which mediates the signalling to p53 upon overexpression of oncogenes such as E1A (de Stanchina et al., 1998) and Myc (Zindy et al., 1998). In contrast, HPV-16 E7 was found to induce p53 even in mouse embryo fibroblasts lacking ARF (Seavey et al., 1999). Interestingly, cells containing wild-type p53 and expressing E7 continue to divide, indicating that E7 is able to overcome p53-induced growth arrest (Hickman et al., 1997), and indeed the induced p53 remains transcriptionally inert in E7-expressing cells (Eichten et al., 2002). However, the induction of p53 by E7 results in p53-dependent apoptosis (Jones et al., 1997b; Hickman et al., 1997; Stoppler et al., 1998), unless E6 is also present to counteract this effect.

HPV E6

The E6 proteins of human papillomaviruses are approximately 150 amino acids long, with an apparent molecular mass of 18 kDa. Figure 3 shows the structure of HPV-18 E6 with the known sites of interaction with some of its cellular target proteins, many of which are also targeted by the transforming proteins of other tumour viruses. A major characteristic of E6 structure is the presence of two zinc fingers formed by Cys-X-X-Cys motifs (Cole and Danos, 1987; Barbosa et al., 1989), whose integrity is essential for virtually all the biological functions of E6 (Kanda et al., 1991; Sherman and Schlegel, 1996), and indeed they are highly conserved throughout all HPV types (Cole and Danos, 1987). A hydrophilic N-terminal domain precedes the zinc fingers that are separated by a hydrophobic region. A unique characteristic of the high-risk mucosal HPV E6 proteins is the presence of a PDZ domain-binding motif at their extreme carboxyl-termini (Kiyono et al., 1997; Lee et al., 1997), which comprises a protein kinase A (PKA) phosphorylation site (Kühne et al., 2000). PDZ (PSD95/Dlg/ZO-1) domains are protein-protein interaction modules found on a large number of cellular proteins involved in both structural and signalling functions, which interact with their protein partners bearing short carboxyl-terminal PDZ-binding motifs (reviewed in Kim, 1997; Harris and Lim, 2001).

Due to its extremely low levels (Androphy et al., 1985; Banks et al., 1987), the subcellular localisation of the endogenous E6 protein has proven extremely difficult to determine. Indeed, different studies have reported E6 to be localised either in the nucleus, cytoplasm or membranes (Androphy et al., 1985; Liang et al., 1993; Sherman and Schlegel, 1996). It has been recently shown that upon overexpression in human keratinocytes, the E6 proteins of low-risk HPVs have a completely different cellular localisation compared to their high-risk counterparts. Indeed, the low-risk HPV-11 E6 is predominantly concentrated in the nucleus, where it accumulates into discrete domains corresponding to PML oncogenic domains (PODs) (Guccione et al., 2002). This feature is intriguing, since targeting viral proteins to nuclear PODs appears to be an important step in viral replication (Everett and Maul, 1994; Muller and Dejean, 1999), and in fact it has been observed that DNA replication of the low-risk HPVs

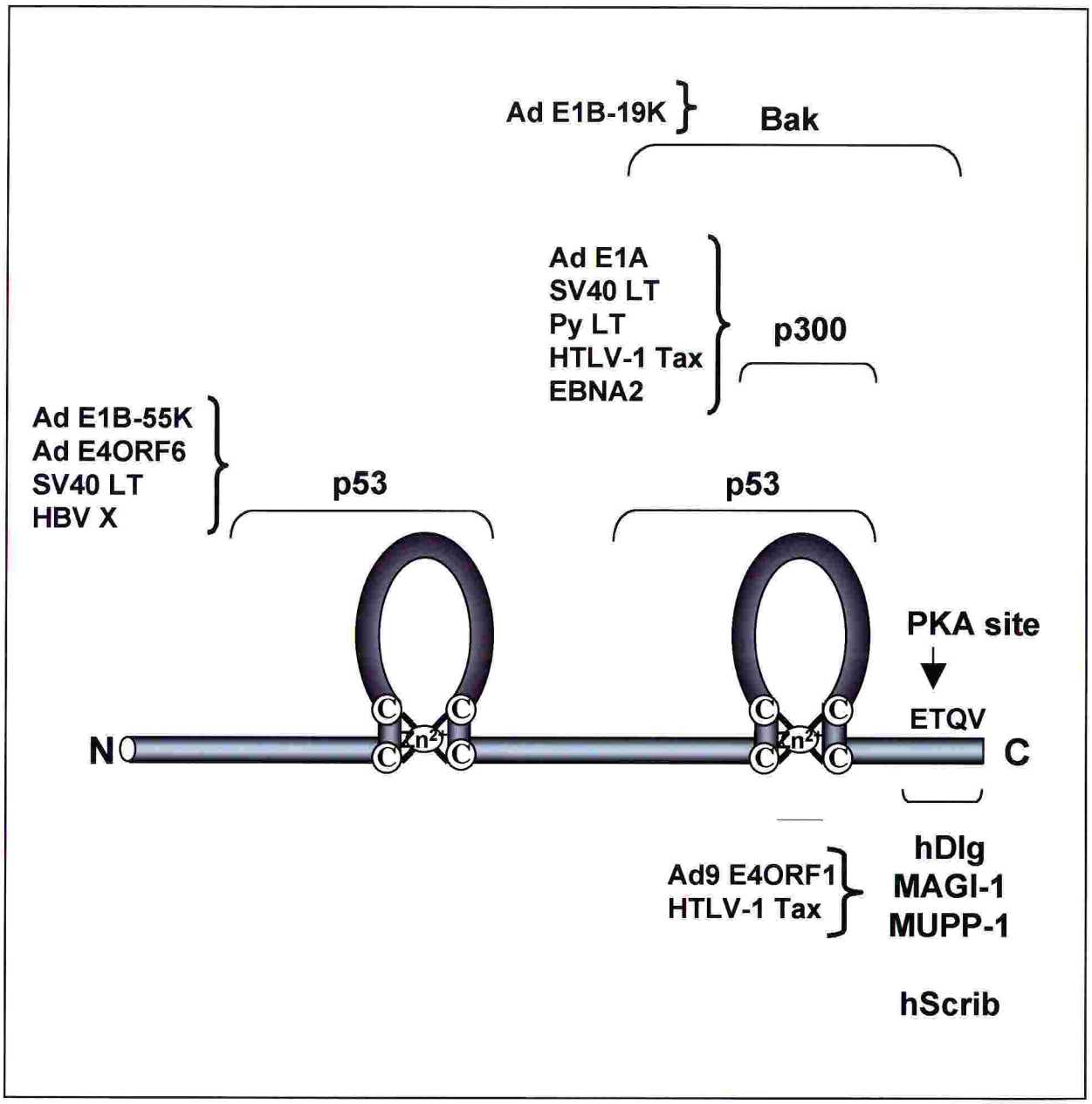


Figure 3. The high-risk HPV E6 protein.
Schematic diagram of E6 showing the two zinc fingers, together with the regions involved in interactions with cellular proteins that are targeted by the oncoproteins of other viruses. Also shown is the C-terminal PDZ-binding motif, ETQV, and the overlapping site of PKA phosphorylation is arrowed.

occurs in the proximity of PODs (Swindle et al., 1999). In contrast, the high-risk HPV E6 proteins fail to colocalise with PODs, and this may provide an explanation as to why the high-risk virus replication cycle is somewhat delayed in comparison to that of low-risk types. Even more striking, while low-risk E6 is mainly nuclear, high-risk E6 proteins are distributed throughout the cell with a high degree of membrane association. This may account for the much higher number of cellular proteins that have been reported to interact with high-risk E6, and it may also explain why high-risk E6 proteins induce many more phenotypic changes within the cell compared with their low-risk counterparts. It has also been speculated that the intracellular localisation of E6 might change, either as a consequence of differentiation or as a result of exogenous stimuli, and there is evidence that this is a highly controlled process. Indeed, proteasome inhibition results in nuclear localisation of high-risk HPV E6. This effect is most likely mediated by nuclear import, since inhibition of nuclear export by leptomycin B has no effect on HPV-18 E6 localisation (Guccione et al., 2002).

Transformation by HPV E6.

The first indirect evidence that E6 was a viral oncoprotein came from studies on cervical tumours and derived cell lines, where E6 was found to be retained and expressed many years after the initial transforming events (Schwarz et al., 1985; Androphy et al., 1987; Banks et al., 1987). Subsequently E6 was found to possess intrinsic transforming activity in a variety of different assay systems. Although E6 has weak transforming activity in established rodent cells (Sedman et al., 1991), high-risk but not low-risk E6 proteins can efficiently cooperate with an activated ras oncogene in the transformation of primary rodent cells (Storey and Banks, 1993; Pim et al., 1994). In addition, E6 has also been found to immortalise primary human mammary epithelial cells at late passage (Band et al., 1991; Wazer et al., 1995), although this activity is also exhibited by the low-risk HPV E6 proteins (Band et al., 1993).

Perhaps the most relevant system for evaluating the transforming potential of the HPV oncoproteins is immortalisation of primary human keratinocytes, which are the

natural host cells of the virus *in vivo*. Numerous studies have shown that high-risk E6 and E7 are together sufficient to induce immortalisation of primary human keratinocytes (Münger et al., 1989b; Hawley-Nelson et al., 1989), while the low-risk HPV proteins are completely inactive in this assay (Woodworht et al., 1989; Pecoraro et al., 1989). It is also interesting to note that although immortalised, these keratinocytes will not form tumours in nude mice. Only following expression of activated oncogenes (DiPaolo et al., 1989), treatment with glucocorticoids (Durst et al., 1989), or after extended passage in tissue culture (Hurlin et al., 1991) do these cells become fully transformed. This nicely resembles the process of HPV induced tumourigenicity *in vivo*, where there are long periods between the initial immortalisation events and the ultimate progression to cervical cancer, thereby highlighting the multistep nature of the disease progression. More recently, efficient models of HPV-induced carcinogenesis have been obtained through generation of transgenic mice. Expression of E6 and E7 together can induce various types of tumours, depending on the particular tissue in which it is targeted (Arbeit et al., 1993; Griep et al., 1993; Comerford et al., 1995). When expression was directed to the basal cells of the squamous epithelium, under control of the human keratin 14 promoter (K14), the transgenic mice developed progressive squamous epithelial neoplasia (Arbeit et al., 1994). When transgenic female mice expressing HPV16 oncogenes downstream of the K14 promoter were subjected to prolonged estrogen treatment, they developed squamous carcinomas of the cervix (Arbeit et al., 1996), thus providing a model for the multistage process of HPV-induced carcinogenesis *in vivo*, and underlying the importance of estrogen hormones as cofactors in the induction of cervical neoplasia. Individual expression of each oncogene induced epithelial hyperplasia and skin tumours (Herber et al., 1996; Song et al., 1999), however E7 was found to primarily cause benign, highly differentiated tumours, whereas those promoted by E6 were mostly malignant. This suggests that the two oncoproteins play different roles in the process of carcinogenesis, and also supports the notion that they act cooperatively to induce transformation. Further investigation was performed both on E6- and on E7-transgenic mice, following treament with specific carcinogens

known to affect distinct stages of tumour formation. Interestingly, E7 was found to primarily cause tumour promotion, whereas E6 contributed weakly to the early stages, while acting more strongly during tumour progression, accelerating the malignant conversion of benign tumours (Song et al., 2000). This finding was particularly important, since it suggests that E6 may be responsible for the malignant progression of HPV-induced tumours *in vivo*, and the metastatic stages of the disease are those which are ultimately fatal.

Ubiquitin-mediated proteolysis.

Several viral proteins, including HPV E6, exploit the ubiquitin-proteasome system for degradation of more abundant cellular substrates, which may interfere with propagation of the virus. Since the study of ubiquitination is crucial to many aspects of the work presented in this thesis, it will be necessary at this point to briefly review this pathway.

Ubiquitin-mediated proteolysis is a highly selective, temporally controlled, and tightly regulated pathway which plays an important role in a broad array of basic cellular processes, including regulation of the cell cycle, modulation of immune and inflammatory responses, control of signal transduction, differentiation and development. All these biological processes involve transition states, which require the fast and irreversible destruction of a single or a specific subset of proteins.

Degradation of a protein via the ubiquitin-proteasome pathway involves an enzymatic cascade that promotes the conjugation of multiple ubiquitin chains to internal lysine residues of the target protein (Ciechanover et al., 2000, for review). This is then recognised by the 26S proteasome, a large multicatalytic protease that selectively degrades polyubiquitinated proteins to small peptides (Voges et al., 1999, for review). Ubiquitin (Ub) is conjugated to target proteins through the carboxyl group of its C-terminal residue (Gly 76) via a three-step mechanism, which begins with the ATP-dependent activation of Ub, through formation of a thiol-ester linkage

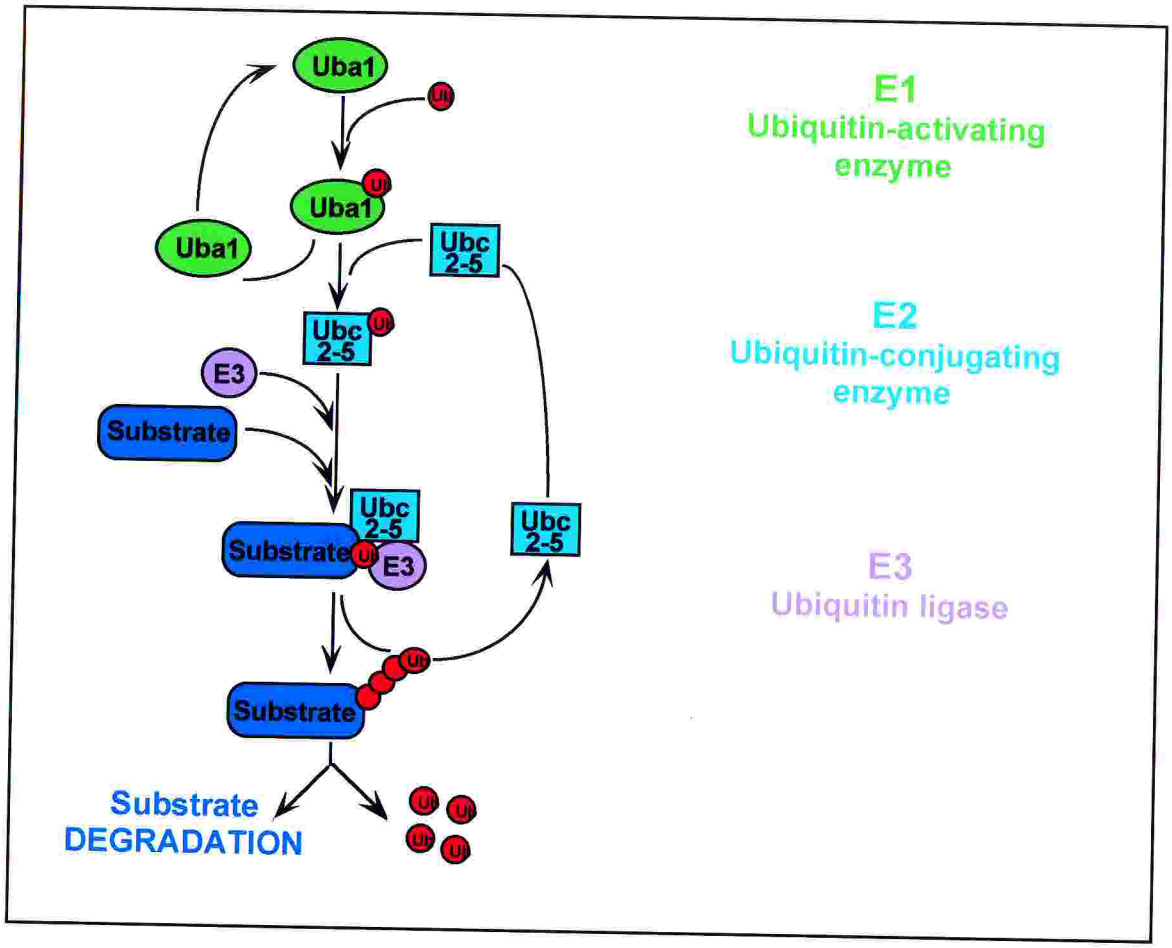


Figure 4. The ubiquitin modification pathway.
Protein modification by ubiquitin (Ub) requires the sequential action of three enzymes: the E1 ubiquitin activating enzyme activates an Ub molecule, the E2 ubiquitin conjugating enzyme then transfers it to the E3 ubiquitin-protein ligase, that in turn catalyses the formation of an isopeptide bond between Ub and the substrate protein. Subsequent Ub molecules are then added on Ub itself, and the resulting polyubiquitinated protein is finally degraded by the proteasome complex while the Ub is recycled.

Adapted from Hodges et al., 1998.

between its C-terminus and the active syte cysteine of the ubiquitin-activating enzyme E1. One of several E2 ubiquitin-conjugating enzymes (Ubcs) then transfers the activated Ub to the substrate that is specifically bound to an E3 ubiquitin-protein ligase. Transfer can either be direct or via an additional E3-ubiquitin thiol-ester intermediate (Figure 4). The structure of the ubiquitin system is hierarchical: a single (or at most a few) E1 species activates all the ubiquitin that is required and transfers it to several E2 isoforms. Each E2 isoform is able to act with either one or several E3 proteins. A limited number of E3 enzymes have been described thus far, but this appears to be a large and rapidly growing family of proteins. E4 has been described recently and is involved in polyubiquitin chain elongation. Its activity, however, appears to be restricted to a limited subset of substrates (Koeg et al., 1999). It is the E3 ligase that provides the high specificity of the system, binding both the E2 and the substrate, and this step can be regulated by cellular pathways. E3s range from single polypeptide chains to large complexes in which substrate recognition and ubiquitin conjugation are relegated to distinct subunits. Multisubunit E3s are often organised in a modular fashion that facilitates the specific recognition of structurally diverse substrates. Most often, the E3 serves as a scaffold protein that brings together the E2 and the substrate. In some cases however, the activated ubiquitin is transferred from E2 to an internal Cys residue on E3 prior to its conjugation to the target: here, the E3 has a catalytic role.

HECT domain proteins (Homologous to E6-AP Carboxyl-Terminus) represent one of two main classes of E3s. They share a conserved catalytic domain, homologous to the C-terminus of the prototypical member of the family, E6-AP (Huibregtse et al., 1995). This domain is 350 amino acid residues long and contains a conserved Cys residue to which the activated ubiquitin moiety is transferred from E2 (Scheffner et al., 1995), forming a thiol-ester intermediate. The large and divergent N-terminal domains mediate substrate recognition (Schwarz et al., 1998). Substrate selection by E6-AP appears to be mediated, at least for its target Mcm7, by a homotypic interaction between enzyme and substrate. This involves a consensus motif, which is present on both proteins and is called L2G box (Kühne and Banks, 1998). Interestingly,

disruption of E6-AP expression in the hippocampal and Purkinje neurons appears to be the cause of the Angelman syndrome (AS), a severe mental retardation and coordination disorder, suggesting that lack of ubiquitination of one or more E6-AP substrates in the brain leads to AS phenotype (Kishino et al., 1997; Matsuura et al., 1997). A critical AS-related substrate, however, has not yet been identified.

A second broad class of E3s is defined by the presence of a RING-finger domain, either on the same polypeptide that recognises the substrate or on a distinct subunit in the context of an E3 complex (Joazeiro and Weissman, 2000, for review). In contrast to HECT E3s, RING E3s do not appear to form thiol-ester intermediates with Ub, while it is likely that RING fingers mediate ubiquitination by facilitating the direct transfer of Ub from E2s to the target Lys. Among them are Mdm2, which ubiquitinates p53 (see below), APC (Anaphase Promoting Complex), which targets mitotic substrates (Sudakin et al., 1995), and the tumour suppressor BRCA-1, whose mutations in the RING domain have been implicated in familial breast and ovarian cancers (Venkitaraman, 2002).

A small noncanonical ring finger protein, Rbx1 (also referred to as ROC1), is an essential component of SCF (Skp1/cullin-1/F-box protein) multicomplex E3 ligases. These are named according to the variable F-box subunit (e.g. $SCF^{\beta-TrCP}$, SCF^{Skp2}) which provides binding specificity to the substrates. F-box proteins anchor to Skp1 through the F-box domain (Bai et al, 1996), while their WD repeats bind selectively phosphorylated substrates. For example, βTrCP has been demonstrated to mediate degradation of IκBα (Yaron et al., 1998) and β-catenin (Kitagawa et al., 1999), by specifically recognising the serine-phosphorylated motif DSGXXS. A very similar consensus site, DSGXXXS, mediates the interaction of β-TrCP with the transcription factor ATF4. Also in this case Ser phosphorylation of the β-TrCP binding site on ATF4 is required to allow its ubiquitination (Lassot et al., 2001). An exception to this rule has however been shown for degradation of p105, the precursor of NF-κB transcription factor, although this represents a unique case in which ubiquitination is involved in limited processing, rather than in complete destruction of a target substrate (Orian et al., 2000). The C-terminal domain of the molecule is degraded

specifically, leaving behind p50, the active subunit of the factor. In this case, cleavage requires three domains: a processing "stop" signal (Lin and Ghosh, 1996), a ubiquitination domain (Orian et al., 1999), and a C-terminal sequence (Heissmeyer et al., 1999) that upon phosphorylation, is recognised by $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase, although not containing a canonical β-TrCP binding site (Orian et al., 2000). Ubiquitination by SCF E3 ligases therefore appears to be modulated through dynamic phosphorylation of the substrates, that affects their recognition by the F-box component. For example, NF-κB activation is achieved through ubiquitin-mediated degradation of its inhibitor IκB. Work by several groups has provided the following model for IκB destruction: a signal transduction cascade, initiated by exogenous stimuli, leads to the activation of an IκB-kinase complex. This phosphorylates IκB on two specific serine residues within the β–TrCP binding site, thereby triggering ubiquitination (Spencer et al., 1999; Yaron et al., 1997). Regulation of the short-lived signal transducer/transcription factor β–catenin follows a very similar route. Upon induction, the GSK3β kinase phosphorylates two Ser residues within the β–TrCP binding site on β–catenin, which is then selectively ubiquitinated by the $SCF^{\beta\text{-}TrCP}$ complex (Kitagawa et al., 1999; Liu C. et al., 1999).

The above described phosphorylation-dependent substrate selection is an example of how the process of ubiquitination can be highly specific, allowing at the same time for dynamic regulation. There are other modes of regulation, depending upon the E3 ligase. For example, phosphorylation of Mdm2 has been proposed to inhibit substrate binding (Mayo et al., 1997), moreover its ligase activity can be modulated by interactions with other proteins such as $p14^{ARF}$ (see below). This effect is thought to involve a conformational change of the RING domain, that also exposes a nucleolar localisation signal, leading to the compartimental segregation of Mdm2 from its substrate p53 (Tao and Levine, 1999a; Weber et al. 1999). Mdm2 activities are also inhibited by dimerisation with its close relative MdmX through their RING fingers (Sharp et al., 1999; Migliorini et al., 2002). Finally ancillary proteins, such as some viral factors, are able to alter the E3 ligase specificity, directing it towards a substrate that would not be normally recognised. Two cases, degradation of p53 by the HPV

E6/E6AP complex (Scheffner et al., 1993) and degradation of CD4 by HIV-1 Vpu/ $SCF^{\beta-TrCP}$ will be discussed below.

Interactions between HPV E6 and p53.

HPV E6 is a multifunctional protein, which efficiently interferes with diverse cellular pathways. Analysis of its cellular targets has provided a wealth of information on how E6 contributes to malignant transformation. The first cellular target of E6 to be identified, and probably still the most important, was p53.

The p53 tumour suppressor.

Although it is not required for normal cell proliferation (Donehower et al., 1992), the tumour suppressor p53 protein plays a fundamental role in orchestrating the cellular response to a variety of genotoxic and cytotoxic stresses which may affect the integrity of the cellular genome and ultimately lead to abnormal cell proliferation. Thus, it is not surprising that about a half of all human cancers select against wild-type p53 function (Hollstein et al., 1996). Neo-synthesised p53 is present within the cell in a latent form, inactive for transcription, and its protein levels are normally kept low in most cells by means of a relatively high turnover rate, to allow physiological cell proliferation and development. Many diverse stress signals such as DNA damage, microtubule disruption, nucleotide depletion, oncogene activation, hypoxia and changes in temperature or pH can trigger signal transduction cascades, which converge on p53 and mediate its activation (Figure 5). This activation involves several different mechanisms that are highly correlated. A complex interplay of post-translational modifications such as phosphorylation/dephosphorylation, acetylation, sumolation and glycosylation, as well as binding to regulatory proteins, lead to p53 stabilisation, nuclear accumulation and activation of sequence-specific DNA binding

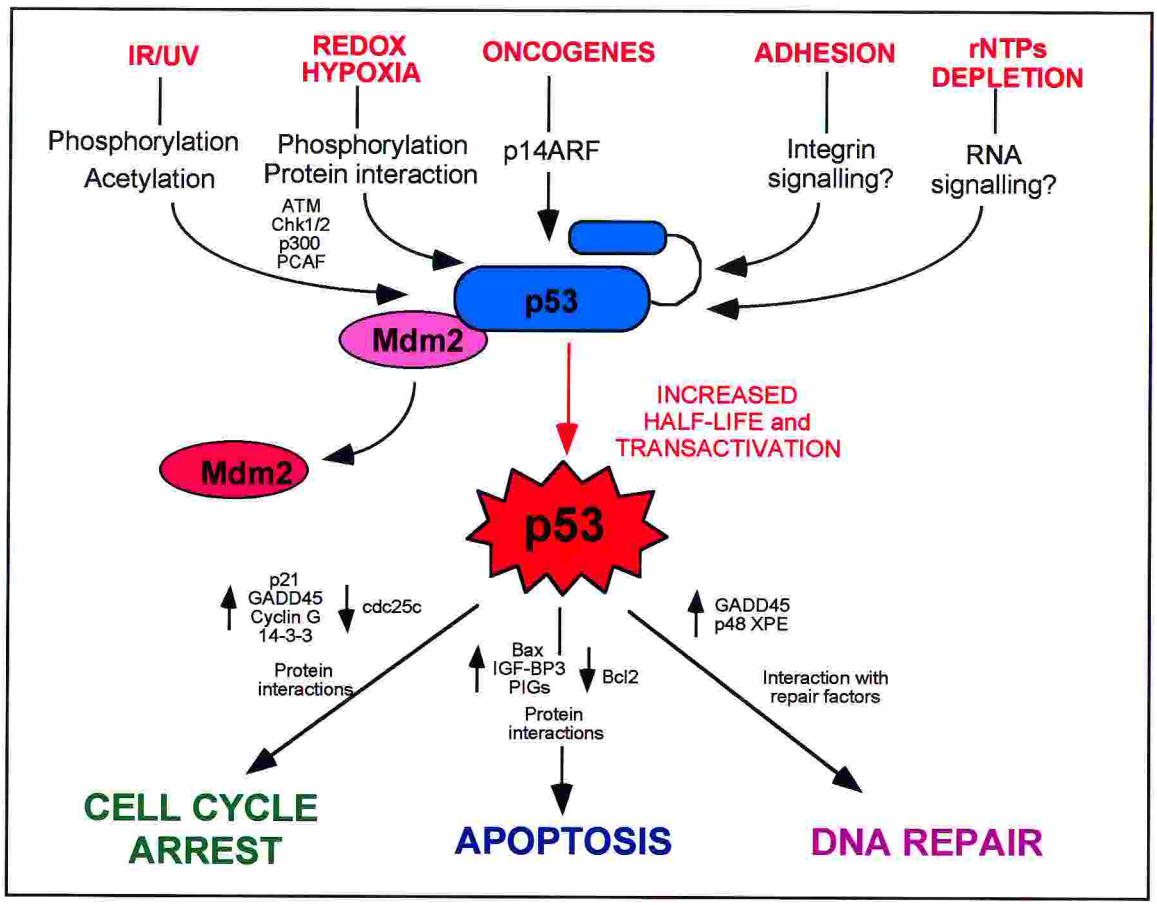


Figure 5. p53 activation pathways.
Different kinds of cellular stress trigger signalling pathways that target p53 with post-translational modifications, resulting in the activation and stabilisation of the protein.
Activated p53 acts as a transcription factor and interacts with several cellular protein partners for modulating its downstream responses.

through conformational changes and tetramerisation (reviewed in Jimenez et al., 1999; Ashcroft and Vousden, 1999).

For instance, DNA-damage induced phosphorylation can contribute to p53 regulation by affecting the binding of co-activators and of the negative regulator Mdm2 (Pise-Masison et al., 1998; Shieh et al., 1997; Shieh et al., 1999). Mdm2 not only interferes with transactivation of p53 target genes in the nucleus (Wu X. et al., 1993), but also stimulates p53 degradation through the ubiquitin-proteasome pathway in the cytoplasm (Kubbutat et al., 1997; Haupt et al., 1997). Interestingly, Mdm2 is also a transcriptional target of p53 (Barak et al., 1993), suggesting the existence of a negative feedback loop, probably required for the termination of the p53 response and recovery from the p53-induced cell cycle arrest. The ability of Mdm2 to promote p53 ubiquitination can be modulated not only by covalent modifications but also by binding to other regulatory proteins like $p14^{ARF}$, that is induced upon oncogene expression (Zindy et al., 1998; de Stanchina et al., 1998) via hyperproliferative signals (Sherr, 1998). $p14^{ARF}$ is a nucleolar protein that has been reported to interact with Mdm2 and, although not interfering with Mdm2 binding to p53, nonetheless prevents p53 proteolysis. This effect can be mediated by ARF through inhibition of the ubiquitin-ligase activity of Mdm2 (Honda and Yasuda, 1999) as well as through its sequestration in the nucleolus, releasing p53 in the nucleoplasm (Weber et al., 1999). Interestingly, a role has been postulated for Mdm2 also in mediating nucleo-cytoplasmic shuttling of p53, adding an extra-level of complexity to the regulation of p53 degradation. Mdm2 contains a functional nuclear export signal and has been proposed to facilitate both p53 export from the nucleus and its degradation (Stommel et al., 1999). Even if there are some controversial data regarding this hypothesis, it is clear that Mdm2 nucleo-cytoplasmic shuttling is required to activate its ubiquitin-ligase function (Freedman and Levine, 1998; Roth et al., 1998; Isaacs et al., 1999; Tao and Levine, 1999b). In addition, it has been demonstrated that the ubiquitin-ligase activity of Mdm2 is critical for the export of p53 from the nucleus (Boyd et al., 2000; Geyer et al., 2000). One possible interpretation of these results is that ubiquitination of p53 by Mdm2, which can occur in the nucleus (Yu et al., 2000), reveals or activates a

nuclear export signal (Stommel et al., 1999), and this hypothesis has been confirmed by recent reports (Lohrum et al., 2001; Gu et al., 2001). p53 nuclear localisation needs to be tightly regulated, being critical for its transcriptional activity. Three lysine-rich nuclear localisation signals within the p53 carboxyl-terminus allow efficient nuclear import of p53, which depends on its interaction with the microtubule network and on its transport towards the nucleus through dynein (Giannakakou et al., 2000). p53 also contains functional nuclear export signals, one within its carboxyl-terminus (Stommel et al., 1999) and a second in the amino-terminus, whose function is inhibited by phosphorylation in response to DNA damage (Zhang and Xiong, 2001). Therefore, DNA damage induced phosphorylation may achieve optimal activation of p53 by both inhibiting its binding to Mdm2 and its nuclear export (Maya et al., 2001).

Once activated, p53 coordinates the cellular stress response by regulating the expression of effector genes involved in mediating growth arrest, DNA repair and apoptosis, or by directly interacting with protein partners (Levine, 1997; Ko and Prives, 1996; Jayaraman and Prives, 1999). The specific nature of the p53 response is largely dependent on the type of stress and on the cellular context, leading to biological activities as diverse as reversible cell cycle arrest, induction of irreversible senescence or apoptotic cell death. Induction of G1 cell cycle arrest is largely based on transcriptional activation of the inhibitor of the cyclin-dependent kinases, p21 (el-Deiry et al., 1993), and also on inhibition of DNA replication, by both p21-dependent and independent mechanisms. Induction of the GADD45 gene by p53 has also been related to cell cycle arrest (Kastan et al., 1992). In addition, p53 is able to block cell cycle progression also through transcription-independent mechanisms. For instance, binding to cyclin H enables p53 to impair the activity of the CDK-activating kinase CAK (Schneider et al., 1998), moreover p53 has been shown to be required for Gas1-dependent growth arrest (Ruaro et al., 1997). There is also evidence that p53 can induce cell cycle arrest at the G2/M transition (Gualberto et al., 1998), possibly through induction of cyclin G (Shimizu et al., 1998), GADD45 (Zhan et al., 1999) and 14-3-3σ effector genes (Hermeking et al., 1997).

Several reports have implicated p53 in direct regulation of DNA repair and replication (Cowell et al., 2000; Sigal et al., 2001; Honma et al., 1997). The products of the p53 target genes p21 and GADD45 interact with PCNA, a factor involved in both DNA repair and replication. Moreover, the p53 C-terminal domain binds with high affinity to DNA double-strand breaks favouring end-joining (Bakalkin et al., 1995; Lee et al., 1995), and indeed p53 harbours intrinsic exonuclease and DNA reannealing activities, as well as being able to interact with several factors involved in DNA repair (Albrechtsen et al., 1999). It has been postulated that in normal, unstressed cells, "latent" p53 is not an inactive protein, but it may be able to perform repair functions, thereby preventing mutations arising from endogenous DNA damage. Under stress conditions however, activation of p53 sequence-specific DNA binding involves a conformational shift that inhibits its exonuclease activity, thereby allowing activated p53 to induce growth arrest and apoptosis. p53-dependent apoptosis is an important mechanism by which transformation is suppressed in oncogene-expressing cells (Symonds et al., 1994), and this may be achieved by p53 either through transcriptional activation or direct protein signalling or a combination of both. In particular, the proline-rich region of p53 has been shown to be required for the induction of apoptosis, probably through the interaction with specific cellular factors (Walker and Levine, 1996). p53 can trigger apoptosis by affecting the Bax/Bcl-2 equilibrium, by inducing the expression of the death effector Bax (Miyashita and Reed, 1995) and repressing the anti-apoptotic gene Bcl-2 (Miyashita et al., 1994). Other p53-responsive genes involved in apoptosis include the death receptor Fas/APO1 (Owen-Shaub et al., 1995), IGF-BP3 that inhibits the mitogenic and survival activities of the IGF receptor (Buckbinder et al., 1995), and the so-called PIG genes, that are involved in generation and response to oxidative stress (Polyak et al., 1997).

A number of factors can affect the decision of a cell to enter either p53-mediated cell cycle arrest or apoptotic pathways. Under conditions in which the DNA is badly damaged, survival factors are limiting, pRB is inactivated or oncogene expression, for example that of HPV E7, is forcing the cell into a replicative cycle, p53-mediated apoptosis prevails.

E6-mediated degradation of p53.

From the above introduction it can be inferred that the p53 tumour suppressor represents a major constraint to viral replication, since, once activated by the unscheduled induction of DNA replication, it can promote cell cycle arrest or apoptosis of the infected cell. To overcome this obstacle, several viruses encode proteins that functionally inactivate p53. SV40 LT prevents transactivation of p53 target genes through association with its DNA binding domain (Ruppert and Stillman, 1993), Adenovirus E1B-55K abolishes the same function by binding to the transactivating domain of p53 (Lin et al., 1994), yet in association with E4orf6 it can also lead to p53 degradation (Steegenga et al. 1998; Querido et al., 2001), while the Hepatitis B virus protein X sequesters p53 in the cytoplasm (Elmore et al., 1997; Takada et al., 1997).

A major strategy employed by the high-risk HPV E6 proteins to abrogate p53's oncosuppressive functions is to induce its degradation through the ubiquitin-proteasome pathway (Scheffner et al., 1990). As a consequence, p53 levels are extremely low in cervical tumour cells (Scheffner et al., 1991; Matlashewski et al., 1986), and p53-induced growth arrest and apoptosis in response to DNA damage are abolished (Kessis et al., 1993; Foster et al., 1994). Indeed, within cervical tumours, unlike most other cancers, p53 is almost invariably wild-type (Crook et al., 1991a; Scheffner et al., 1991), perhaps indicating that the effects of E6 are analogous to an inactivating mutation. It has been reported that in HPV-positive cancer cells degradation of p53 depends entirely on E6, while the Mdm2-dependent pathway is inhibited (Hengstermann et al., 2001). This indicates that E6 can target p53 for degradation under conditions when this would be normally blocked, e.g. after DNA damage, thereby allowing the accumulation of genomic mutations in the infected keratinocytes, and thus contributing towards malignant progression. E6 reactivates degradation of p53 by recruiting the prototype HECT-domain ubiquitin ligase: E6AP (see above) (Scheffner et al., 1993), which normally is not involved in the ubiquitination of p53. High-risk HPV E6 binds to E6AP within its N-terminal

substrate recognition domain (Huibregtse et al., 1993a), and formation of a stable E6-E6AP complex precedes association with p53, thereby redirecting the substrate specificity of E6AP towards p53, which is unable to bind E6AP independently of E6 (Huibregtse et al., 1993b). Indeed, approaches aimed at blocking E6AP activity, either by the use of antisense oligonucleotides (Beer-Romero et al., 1997; Traidej et al., 2000) or dominant negative mutants (Talis et al., 1998), increased the levels of p53 in HPV-positive, but not in HPV-negative cells, confirming that E6AP plays an essential role in E6 directed degradation of p53 *in vivo*, but has no effect on p53 levels in cells lacking E6. The efficiency in mediating degradation of p53 varies between different E6 proteins, depending on their ability to interact with both p53 and E6AP. Both high- and low-risk mucosal HPV E6 proteins are able to bind the p53 C-terminus, however such interactions do not induce degradation. Binding to the core region of p53 is much greater for high-risk E6 proteins and is enhanced by the presence of E6AP, and it is this interaction that allows efficient degradation of p53 (Li and Coffino, 1996). In addition, HPV-16 E6 binds E6AP more strongly and concomitantly degrades p53 more effectively than HPV-18 E6. HPV-11 E6 has minimal levels of binding to E6AP (Huibregtse et al., 1993b), and degrades p53 *in vivo* only weakly (Storey et al., 1998). Interestingly, the E6 proteins of both high- and low-risk cutaneous HPV types do not associate with either E6AP or p53 and are incapable of affecting p53 stability (Elbel et al., 1997), therefore the mechanism by which these viruses evade the constraints that p53 places on viral replication remains to be determined.

Recently it has been reported that, despite binding to different regions of p53, both HPV-16 E6 and E1B55K/E4ORF6 appear to require the same sequence element of p53 to target it for degradation (Gu et al., 2001). This same sequence, comprising residues 92 to 112, has previously been shown to be required also for Mdm2-mediated degradation, possibly functioning as a determinant for p53 stability (Gu et al., 2000). Substitution or deletion of this sequence element of p53 resulted in resistance to degradation by viral proteins. Moreover, swapping the oncoprotein-binding domains together with the p53 residues 92-112 rendered its homolog p73 susceptible to viral protein-mediated degradation.

Effects of E6 upon cell cycle.

Transformed cells expressing HPV-16 or HPV-18 E6 lose the G1 checkpoint very early, presumably due to the degradation of p53 (Dulic et al., 1994; Slebos et al., 1995), and are also resistant to p53-induced growth arrest and apoptosis as a result of DNA damage (Kessis et al., 1993; Foster et al., 1994; Pan and Griep, 1995; Thomas et al., 1996). The G2 checkpoint is initially unaffected (Paules et al., 1995) but there is increased chromosomal instability in E6-expressing cells over time, and this is probably caused by the observed attenuation of the G2 checkpoint function (White et al., 1994; Kauffmann et al., 1997). This may be caused by E6 altering the cyclin/cdk complexes (Xiong et al., 1996), or may relate to the ablation of the p53-regulated G2/M checpoint. In normal diploid human cells expressing HPV-16 E6, the G2 delay in response to ionising radiation is considerably reduced, probably owing to the observed increase in the concentrations and activities of cdc2, cyclin A and cyclin B. In these cells, also the checkpoint coupling M-phase entry to the completion of DNA replication and the mitotic spindle checkpoint are disregulated (Thompson et al., 1997; Thomas and Laimins, 1998).

Additional pathways of E6 interference with p53 activities.

Although targeting p53 for degradation is the major route by which E6 overcomes its biological effects, several reports clearly indicate that E6 proteins make use of additional pathways to abrogate p53 growth suppressive activities. Both low- and high-risk HPV E6 proteins are capable of abolishing p53-mediated transcriptional repression *in vivo* (Lechner et al., 1992) and this is likely to occur through binding to the p53 C-terminus (Li and Coffino, 1996). Moreover, the capacity of the high-risk E6 proteins to abrogate transactivation of p53 target genes does not only depend on p53 destabilisation, since E6 mutants defective for degradation retain the ability to abrogate transcriptional activation by p53 *in vivo* (Pim et al., 1994). Several mechanisms can be invoked to explain this property of E6. First, E6 can interfere with the binding of p53 to its DNA recognition site (Lechner and Laimins, 1994; Thomas et

al., 1995). In addition, repression of p53-responsive promoters could be mediated through the interaction of high-risk HPV-16 E6 with the transcriptional coactivators p300/CBP (Patel et al., 1999; Zimmermann et al., 1999), similarly to what has been reported for Adenovirus E1A (Somasundaram and El-Deiry, 1997). Finally, cytoplasmic sequestration of p53 is another common strategy adopted by different viral proteins, such as Adenovirus E1B 55K (König et al., 1999) and HBV protein X (Elmore et al., 1997). This is sufficient to interfere with p53 transcriptional activities, and indeed the HPV E6 proteins also appear to be capable of promoting the nuclear exclusion of p53 (Freedman and Levine, 1998; Vogt Sionov et al., 2001; this thesis).

Regulation of E6-mediated p53 degradation by the E6 proteins.*

Despite the above discussion, it is quite clear that during papillomavirus infection and in HPV-induced cervical lesions not all p53 is degraded, as several studies have reported detectable levels of p53 in HPV-infected cells (Cooper et al., 1993; Lie et al., 1999; Vassallo et al., 2000; and this thesis). A possible viral pathway for regulating E6 activity with respect to p53 relies on a series of polypeptides termed E6*, which are expressed exclusively by the high-risk HPV types through alternative splicing of E6 mRNA (Schneider-Gädicke et al., 1988). Interestingly, HPV-18 E6*I was found to interact with both the full length E6 and E6AP, thereby blocking degradation of p53 (Pim et al., 1997): this might allow a fine-tuning of the activity of E6 with respect to p53 during viral infection.

Interestingly, while p53 has been shown to specifically inhibit HPV and BPV-1 amplificational DNA replication *in vivo*, it did not not affect episomal maintenance, which occurs in synchrony with the cell cycle (Lepik et al., 1998). In order to elicit a productive infection, viral DNA amplification needs to be controlled and it is plausible that the activity of E6* could ensure the presence of a limited amount of p53 at the replication sites, where it could both prevent overreplication of the viral genome and, possibly, assist DNA synthesis by means of its proofreading capacity. Indeed, HPV recruits DNA polymerase α for DNA replication, and the 3'-5'

exonuclease activity of p53 could enhance the replicative fidelity of this enzyme (Huang, 1998). It is interesting to note that p53 has been found in the replication centres of several viruses such as HSV (Wilcock and Lane, 1991), CMV (Fortunato and Spector, 1998) and Adenovirus (König et al., 1999), and recent studies reported an interaction between the HPV ori binding protein E2 and p53, further suggesting a potential positive role for p53 in viral replication (Massimi et al., 1999).

Polymorphic variants of p53 and E6.

A common p53 polymorphism results in either a proline or an arginine at residue 72 (Matlashewski et al., 1987), the Arg variant disrupting one of the five PXXP SH3-binding domains of the polyproline region, which is involved in the induction of apoptosis (Walker and Levine, 1996). A functional analysis has shown that, while p53 Pro is a stronger transcriptional activator, p53 Arg is more effective in preventing immortalisation of primary rodent cells, an activity which correlates with the induction of apoptosis (Thomas M. et al., 1999). Interestingly, the Arg variant is significantly more susceptible than the Pro counterpart to HPV-18 E6 mediated degradation *in vivo*, and it can be targeted for proteolysis also by the low risk HPV-11 E6 protein, while the Pro form cannot (Storey et al., 1998). From an evolutionistic point of view, it could be speculated that the E6 proteins have adapted to degrade more effectively the Arg variant of p53, which causes a stronger apoptotic response to virus-induced cellular proliferation. These data are supported by genetic studies indicating a correlation between the homozygous p53 Arg genotype and the risk of developing HPV-associated cervical cancer (Storey et al., 1998; Zehbe et al., 1999). However, the data on a possible association of p53 polymorphisms with cervical cancer risk are still conflicting (Lanham et al., 1998; Giannoudis et al., 1999), therefore analyses based on a more precise classification of specimens with respect to HPV types would appear necessary. Since many intratypic HPV variants have also been described (Bernard et al., 1994), the problem may be further complicated by the presence of different forms of the E6 protein, which vary significantly with respect to

activities such as transformation and degradation of p53 (Conrad-Stöppler et al., 1996), with consequent clinical implications (Zehbe et al., 1998).

p53-independent activities of E6.

Although p53 is a vital aspect of E6 function, analysis of E6 mutants has shown that activities other than p53 degradation are required for its full transforming potential (Nakagawa et al., 1995), and several reports have described mutants of E6 which have lost the ability to interact with p53 while retaining the capacity to transform cells (Pim et al., 1994; Liu Y. et al., 1999). Moreover, the oncogenic potential of cutaneous HPVs mainly relies on their E6 proteins which, however, lack any interaction with p53 or E6AP, and fail to induce ubiquitination and degradation of p53 (Elbel et al., 1997). It is now clear that, in common with many viral oncoproteins, E6 is a multifunctional protein and, in line with the above observations, numerous cellular targets have recently been identified (Table 2). There is now active debate as to which of these other activities of E6 contributes more towards the development of malignancy.

De-regulation of transcription and DNA replication by HPV E6.

The E6 proteins of both high- and low-risk HPV types have long been known to modulate transcription from many cellular and viral promoters (Sedman et al., 1991; Desaintes et al., 1992; Veldman et al., 2001). It is only recently, however, that an understanding of some of the mechanisms by which this occurs has come with the demonstration that E6 interacts with p300/CBP (Patel et al., 1999; Zimmermann et al., 1999, 2000). The p300/CBP transcriptional co-activators play important roles in activating a great number of genes involved in the regulation of the cell cycle, differentiation and the immune response. Indeed, many viral oncoproteins including SV40 LT, Polyomavirus LT, EBNA2, Adenovirus E1A and HTLV-1 Tax have been shown to require the interaction with p300 for optimal transforming activity

(Goodman and Smolik, 2000, for review), highlighting its central importance in regulating the cellular homeostasis. Both low- and high-risk HPV E6 proteins were shown to directly bind to an extended region of p300/CBP (Zimmerman et al., 1999; Patel et al., 1999; Bernat et al., 2002). Interestingly, when tested in primary epithelial cell-transformation assays, both high-risk and low-risk HPV E6 proteins were able to complement a mutant Adenovirus E1A protein defective for p300 binding, while an E6 mutant unable to bind p300 was not capable of complementing E1A defect (Bernat et al., 2002). This suggests that the E6-p300 interaction is required for the ability of E6 to contribute towards cell transformation. Indeed, HPV-16 E6 was also shown to inhibit the intrinsic transcriptional activity of p300/CBP on both p53- and NFκB-responsive promoter elements (Zimmermann et al., 1999; Patel et al., 1999). Obviously, p300/CBP affects the expression of many different cellular genes, inlcuding those regulating differentiation (Bannister and Kouzarides, 1995; Goodman and Smolik, 2000), and at present it is not clear which are the true targets for E6-mediated inhibition via the p300/CBP interaction. However, E6 interference at NFκB-responsive promoters is particularly intriguing, since disregulation of the NFκB pathway can result in hyperproliferation of the stratum spinosum, the epithelial layer in which HPV DNA amplification occurs (Hu et al., 1999; Takeda et al., 1999). NFκB is activated upon infection by a number of viruses, including HIV-1 (DeLuca et al., 1996), HTLV-1 (Munoz et al., 1994), HBV (Meyer et al., 1992) and HPV (Nees et al., 2001; L. Havard personal communication), and promotes transcription of a number of genes involved in the local immune response such as class I MHC, interleukins and GM-CSF, some of which are synthesised directly by keratinocytes (Baldwin, 1996; Tomic-Canic et al., 1998, for review). Therefore inhibition of the NFκB pathway may help the virus to escape immune recognition. Moreover, virus-induced activation of NFκB stimulates the IFN-β promoter (Thanos and Maniatis, 1995), and therefore E6 transcriptional interference could also serve to block the interferon-mediated antiviral response, which inhibits viral replication and proliferation of the infected cells. Interestingly, HPV-16 E6 has also been found to bind the interferon regulatory factor 3 (IRF-3) (Ronco et al., 1998), which is an important transactivator of interferons and

binds to the regulatory elements of the IFN-β promoter (Wathelet et al., 1998). E6 binding to IRF-3 does not result in ubiquitination or degradation, however HPV-16 E6 expression was able to block the induction of IFN-β after a viral infection (Ronco et al., 1998). It has been discovered that in response to viral infection the assembly and activation of a large transcription factor complex, which includes IRF-3, CBP and p300 and acts as a positive regulator of α/β interferon-responsive genes is stimulated (Weaver et al., 1998). It is likely that the combined targeting of all these components by E6 will contribute to disruption of the cellular antiviral response. Interestingly, a recent screen of cDNA microarrays demonstrated that transfection of HPV-16 E6 in differentiating cervical keratinocytes also downregulates the expression of interferon-responsive genes, further supporting this notion. However, this was also reported to upregulate NFκB responsive genes (Nees et al., 2001). Finally it should also be borne in mind that the E6/p300 interaction, and its possible contribution towards survival of the transformed cell, could be a by-product of the regulation of viral gene expression. Thus, the HPV major transcriptional activator, E2, also interacts with p300/CBP (Lee D. et al., 2000). This appears to involve a cellular protein, AMF-1/Gps2, which in turn enhances p300 activity but which, intriguingly, is also a target for E6-mediated degradation (Peng et al., 2000; Degenhardt and Silverstein, 2001). Therefore the interaction between E6 and p300 may represent a means of downregulating E2 transcriptional activity through a feedback mechanism.

It has been recently reported that both high- and low-risk HPV E6 proteins interfere with the transcriptional activity of the p53 homolog p73 (Park et al., 2001). However, several studies have reported that p73 is unable to bind HPV E6 proteins and is not susceptible to E6-dependent proteolysis (Marin et al., 1998; Gu et al., 2001; Park et al., 2001; Prabhu et al., 1998), therefore transcriptional interference by E6 would appear to be indirect, possibly as a result of its interaction with p300.

An additional link with tumour progression has come from studies showing deregulation of the cellular DNA replication machinery by HPV E6. Normal somatic cells terminate their replicative life span through a pathway leading to cellular senescence, which is triggered in response to critically shortened telomere DNA.

Neoplastic cells must first overcome the senescence checkpoint mechanisms, and subsequently activate telomerase to propagate indefinitely. In senescent cells the levels of $p16^{ink}$ are elevated, and its inactivation has been shown to abrogate a late step during senescence of human epithelial cells (Kiyono et al., 1998). Interestingly, the immortalisation of human uroepithelial cells by HPV-16 E6 correlated with undetectable levels of $p16^{ink}$ due to gene inactivation. In addition, telomerase activity is absent from most normal somatic cells and its induction is a hallmark of immortalisation and malignant transformation (Kim et al., 1994). Interestingly, activation of telomerase is detected in cervical carcinomas and in a subset of high grade cervical lesions associated with high-risk HPV (Snijders et al., 1998; Nair et al., 2000), and it has been demonstrated that it can be induced by HPV-16 E6 in primary epithelial cells through a p53-independent mechanism (Klingelhutz et al., 1996; Veldman et al., 2001). A recent study reported that this is due to E6-mediated transcriptional activation of the gene encoding the telomerase catalytic subunit, hTERT, and the minimal promoter region involved in induction by E6 was also mapped (Veldman et al., 2001; Oh et al., 2001). It is possible however that this represents an indirect activity of E6, since it is unlikely that hTERT activation will be relevant to viral infection and replication.

E6 proteins of both benign and oncogenic HPV types have also been found to interact with hMcm7, a component of the DNA replication licensing complex, suggesting that this interaction might be required for viral genome replication (Kukimoto et al., 1998; Kühne and Banks, 1998). However, hMcm7 is a substrate for E6AP-dependent ubiquitination, and indeed HPV-18 E6 was able to enhance its proteasome degradation *in vivo* (Kühne and Banks, 1998): this might cause p53-independent chromosomal abnormalities in HPV-positive cells.

target protein	high-risk E6	low-risk E6	degradation	cellular function
p53	yes	yes	yes	tumour suppressor, apoptosis inducer
E6AP	yes	no	yes	ubiquitin ligase
hMcm7	yes	yes	yes	DNA replication initiation
E6TP1	yes	yes	yes	putative GAP protein
Bak	yes	yes	yes	apoptosis inducer
c-Myc	yes	no	yes	transcription factor
p300/CBP	yes	yes	no	transcriptional coactivators
AMF-1/Gps2	yes	yes	yes	transcriptional coactivator
IRF-3	yes (HPV-16)	no	no	transcription factor
E6BP/ERC-55	yes	no	no	Ca^{++} binding protein
paxillin	yes	no	no	signal transduction
hDlg	yes	no	yes	control of cell polarity and growth
MAGI-1	yes	no	yes	putative signal transduction
MUPP-1	yes	no	yes	scaffolding protein/ putative signal transduction
hScrib	yes	no	yes	control of cell polarity and growth

Table 2. Cellular targets of HPV E6.

Mitogenic activities of E6.

It has been shown that expression of oncogenic E6 proteins can affect the early stages of carcinogenesis in transgenic mice, although to a lesser extent than E7 (Song et al., 2000), and this probably relies on the ability of E6 to induce cellular hyperproliferation and epidermal hyperplasia (Song et al., 1999). Interestingly, this would appear to be largely independent of p53, since similar levels of proliferation are induced by E6 in mice which are null for p53 (Song et al., 1999). Several activities of E6 are potentially involved in deregulation of mitogenic pathways. For example E6TP1, a protein found to interact with E6 in a two-hybrid screen, shows high homology with a family of GTPase activating proteins (GAPs) that are negative regulators of Rap (Gao et al., 1999). Therefore E6TP1 might be involved in the inhibition of Rap-mediated mitogenic signalling. Moreover, its gene has been mapped to a putative tumour suppressor locus on chromosome 14 (Menon et al., 1997). High-risk and, to a lesser extent, also low-risk HPV E6 proteins are able to bind E6TP1, while only the oncogenic E6s can promote its degradation. Interestingly, E6AP was reported to participate in the interaction (Gao et al., 1999), and indeed it appears to be responsible for ubiquitination of E6TP1 (Gao et al., 2002). There is a strong correlation between the ability of HPV-16 E6 mutants to degrade E6TP1 and to immortalise human mammary epithelial cells (Gao et al., 2001), however more data concerning the GAP function of E6TP1 would be necessary to understand its role in E6-induced malignancy.

Inhibition of apoptosis.

Both the E6 and E7 oncogenes are capable of independently transforming cells, however their coexpression strongly increases their transforming potential in primary keratinocytes, indicating functional cooperativity (Hawley-Nelson et al., 1989; Münger et al., 1989b). The cells react to abnormal proliferation stimuli, such as those exerted by the E7 oncoprotein, by inducing apoptosis (Pan and Griep, 1994; Bates et al., 1998), and the E6 multifunctional protein appears to be required to block this cellular response (Pan and Griep, 1995). Indeed, intracellular targeting of the HPV-16 E6 protein by E6-binding peptide aptamers resulted in selective apoptotic elimination of HPV-positive, but not HPV-negative cancer cells (Butz et al., 2000). This finding provides direct evidence that the antiapoptotic activity of E6 is required for cancer cell survival. The oncogenic HPV E6 proteins induce degradation of p53 thereby counteracting p53-dependent apoptosis in the infected cell. However, there is increasing evidence that E6 can also inhibit p53-independent apoptotic pathways, promoted by different stimuli. Indeed, in transgenic mice expressing HPV-16 E6 in the ocular lens, E6 was found to prevent apoptosis both in wt and in p53-null animals (Pan and Griep, 1995), and similarly it has been reported to inhibit drug-induced apoptosis in cells lacking p53 (Steller et al., 1996). Among the newly discovered cellular targets of E6, there are indeed several proapoptotic factors. One of them is Bak, whose high levels of expression in the upper epithelial layers (Krajewski et al., 1996) appear to represent a common obstacle for a broad range of HPV types that replicate in differentiating keratinocytes. Indeed, the E6 proteins of both high and low-risk mucosal HPVs, plus those of high-risk cutaneous types have been shown to inhibit Bak-induced apoptosis (Thomas and Banks, 1998; Thomas and Banks, 1999; Jackson and Storey, 2000). HPV-18 E6 has been shown to stimulate the ubiquitin-dependent degradation of Bak catalysed by E6AP, probably by accelerating a normal cellular process, since Bak appears to be a natural substrate of E6AP even in the absence of E6 (Thomas and Banks, 1998). Degradation of Bak by HPV-11 E6 is less effective, and this correlates with a weaker anti-apoptotic activity of the low-risk

mucosal HPV types (Thomas and Banks, 1999). Recently, a link between the infection by cutaneous HPVs and the induction of HPV-associated skin cancer by UV radiation was demonstrated, since the cutaneous E6 proteins were shown to abrogate both p53-dependent and independent apoptosis in response to UV damage. p53 protein levels were unaffected in cells expressing cutaneous E6s, and its transcriptional activity with respect to p21 also did not change (Jackson and Storey, 2000). The mechanism used by these E6 types to inhibit p53-dependent apoptosis is not known, one possibility would be that they mediate the specific inhibition of p53 target genes directly involved in apoptosis, such as the PIG genes induced by DNA damage (Polyak et al., 1997). Moreover, the cutaneous HPV E6 proteins abrogate Bak function by promoting its ubiquitin-mediated degradation. Indeed, Bak protein is undetectable in HPV-positive skin cancers, in contrast to HPV negative cancers, which express it (Jackson et al., 2000). Interestingly, Ad E1B 19K also inhibits Bak induced apoptosis (Farrow et al., 1995; White, 2001), demonstrating a high degree of conservation of function among these different viruses.

The c-Myc transcription factor is normally degraded through the proteasome pathway by two different ubiquitin-conjugating systems. It was shown that expression of high-risk, but not low-risk HPV E6 accelerates the degradation of c-Myc by recruiting, as in the case of p53, the E6AP ubiquitin ligase (Gross-Mesilaty et al., 1998). It may seem unusual that E6 targets a protein that would normally stimulate cell proliferation, but this finding can be also viewed in the light of c-Myc proapoptotic activity. TGFβ is a potent growth inhibitor for many cell types, including most epithelial cells (Lyons and Moses, 1990, for review), and its effects are mediated at least in part by suppression of *c-myc* transcription (Pietenpol et al., 1990a). It has been demonstrated that HPV-16 E7 and other viral proteins binding pRB, such as SV40 LT and Adenovirus E1A, are able to block TGFβ-mediated downregulation of c-Myc, in order to sustain proliferation of the infected keratinocytes (Pietenpol et al., 1990b). However, deregulated expression of c-Myc in differentiating cells induces apoptotic cell death as a control mechanism (Askew et al., 1991). It is therefore

possible that degradation of c-Myc by E6 would contribute towards maintaining the equilibrium between E7-promoted proliferation and cell survival.

Interference with epithelial organisation and differentiation.

High-risk HPV types have evolved to replicate in the differentiated layers of the squamous epithelium that are not permissive for reproduction of the low-risk types. Indeed, a characteristic of the high-risk E6 proteins is their ability to block terminal differentiation of epithelial cells, which normally leads to keratinisation and subsequent cell death. HPV-16 E6 was reported to impair cell differentiation in the ocular lens of transgenic mice (Pan and Griep, 1994). Moreover, expression of HPV-16 E6 caused an expansion of the undifferentiated compartment of the epithelia in K14-E6 transgenes, indicating that the normal differentiation program was delayed; interestingly, this activity of E6 appears to be p53-independent, since it was also observed in p53-null, E6-transgenic mice (Song et al., 1999). Consistent with this is the demonstration that HPV-16 E6 increases the resistance of human keratinocytes to serum and calcium induced differentiation through p53-independent pathways (Sherman et al., 1997; Sherman and Schlegel, 1996; Sherman et al., 2002). Very little is known, however, about the mechanisms by which E6 interferes with keratinocyte differentiation, although this is of great interest being closely related to the malignant potential of the tumours associated with oncogenic HPVs. HPV-16 E6 was reported to interact in a two-hybrid screen with a protein called E6BP (Chen et al., 1995), which is identical to ERC-55, a putative calcium binding protein localised in the endoplasmic reticulum (Weis et al., 1994). Upon expression of E6, E6BP was found to form a complex with both E6 and E6AP *in vivo*, however direct binding to E6AP and E6-targeted degradation were not shown (Chen et al., 1995). Epithelial differentiation is responsive to Ca^{2+} mediated signalling, and it might be speculated that E6 targeting of E6BP contributes to the alteration of terminal differentiation. However, additional targets of E6 are likely to be implicated, since certain E6 mutants have been shown to

be impaired in this activity, while retaining high levels of binding to E6BP (Sherman et al., 2002). It should also be noted that Ca^{2+} signalling has a role in blocking apoptosis, and it is interesting that the antiapoptotic Ad E1B 19K protein also interacts with a putative calcium binding protein localised to the nuclear envelope and ER (Boyd et al., 1994). Hence, the E6-E6BP interaction might similarly be involved in E6-mediated inhibition of p53-independent apoptosis in HPV infected cells.

Cellular adhesion to the extracellular matrix affects many different cellular processes including cell morphology, proliferation and migration. The restriction of cell proliferation to matrix-interacting cells serves to prevent dysplasia, and the circumvention of anchorage dependence plays an important role in tumorigenesis (Sastry and Horwitz, 1996). Cell-matrix adhesion is mediated by specialised structures called focal adhesions, that contain integrins, vinculin, focal adhesion kinase and paxillin (Sastry and Burridge, 2000). Paxillin is involved in mediating signalling from the plasma membrane to focal adhesions and to the actin cytoskeleton, and its activity is regulated by tyrosine phosphorylation in response to various stimuli, including integrin crosslinking and treatment with growth factors (Turner, 2000). Recent studies have shown that HPV-16 E6 binds paxillin and this interaction correlates with E6 transforming activity, although E6 does not appear to target paxillin for degradation (Tong and Howley, 1997). However, since most biological effects of this interaction have been determined for the more highly abundant BPV-1 E6 protein (Tong and Howley, 1997), its biological relevance for high-risk HPV E6 proteins remains to be determined.

Interactions with PDZ proteins: interference with cell-cell adhesion, polarity and proliferation control.

A striking feature of all E6 proteins derived from the high-risk mucosal HPV types is the presence of a highly conserved C-terminal domain, which is not involved in p53 binding and degradation (Crook et al., 1991b; Pim et al., 1994), but which nonetheless contributes to E6 transforming activity, since its deletion impairs E6's ability to transform rodent cells (Kiyono et al., 1997) and immortalise keratinocytes (C. Meyers, personal communication). This region contains a PDZ-binding motif (XT/SXV), a short stretch of amino acids which mediates the specific interaction with proteins containing PDZ domains (Doyle et al., 1996; Songyang et al., 1997). These are 80-90 amino-acid motifs, present on a variety of proteins involved in clustering ion channels, signalling enzymes, and adhesion molecules to specific structures at the membrane-cytoskeleton interface of polarised cells (reviewed in Kim, 1997). An increasing number of PDZ domain-containing proteins, which are involved in the organisation of epithelial architecture were reported to be targeted by the high-risk HPV E6 proteins.

The first PDZ-protein shown to be a target for high-risk E6 was hDlg (Kiyono et al., 1997; Lee et al., 1997). This is the human homologue of the Drosophila tumour suppressor Dlg, required for formation of adherens junctions and for regulation of cell adhesion, apicobasal polarity and proliferation in epithelial tissues. Indeed, mutations causing loss of Dlg functions result in aberrant morphology and invasive growth of epithelial cells, causing embryonic lethality (Woods et al., 1996; Bilder et al., 2000). Recently, a *Dlg* truncating mutation has also been described, which impairs morphogenesis during murine development, resulting in perinatal death (Caruana and Bernstein, 2001). Human Dlg (Figure 6) belongs to the MAGUK (Membrane-Associated-Guanylate-Kinase) family of multidomain proteins and contains an N-terminal proline-rich SH3-binding domain, three PDZ domains, an SH3 domain and a C-terminal guanylate kinase homology domain. hDlg colocalises with E-cadherin at adherens junctions of epithelial cells, (Reuver and Garner, 1998; Ide et al., 1999) and is

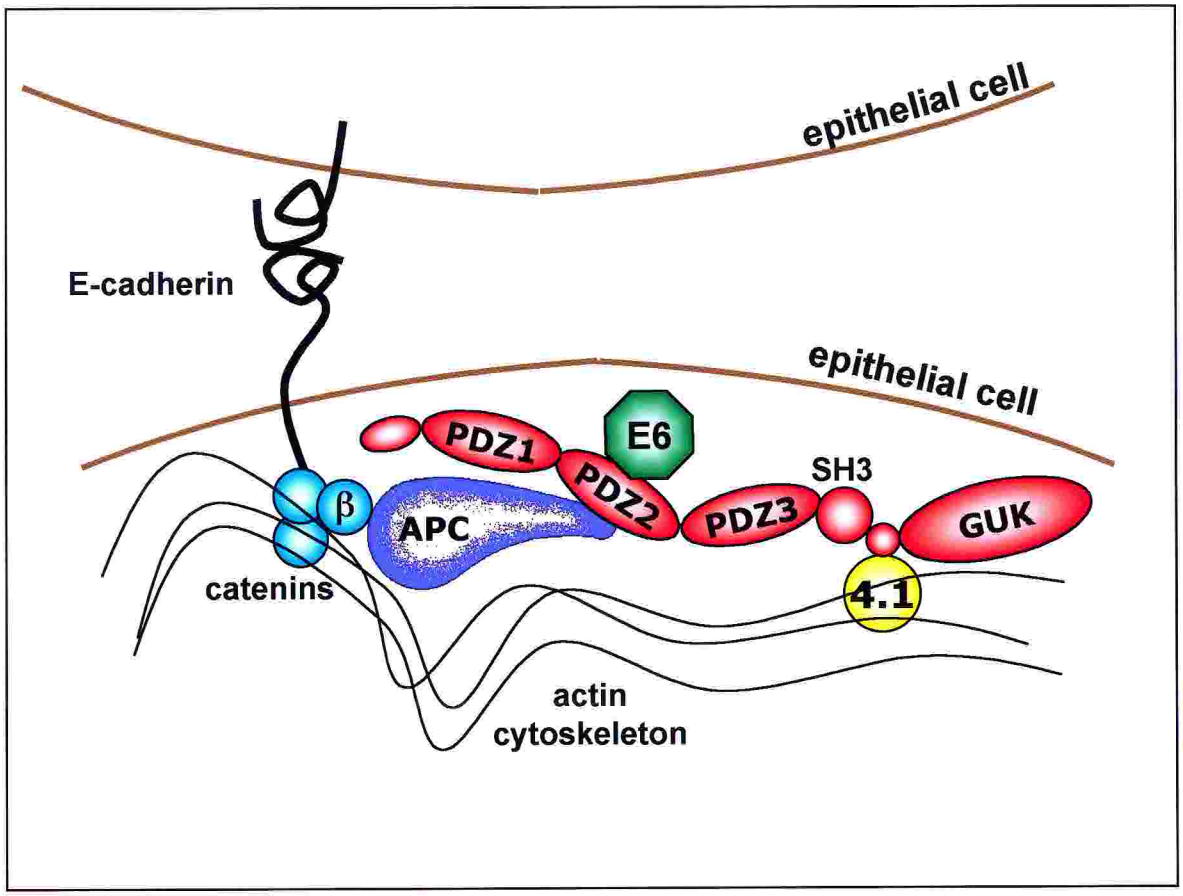


Figure 6. The hDlg protein.

Schematic diagram showing the location of hDlg at the membrane-cytoskeleton interface of epithelial cells at regions of cell-cell contact. hDlg (red) contains 3 PDZ domains, an SH3 signaling domain and a Guanylate Kinase (GUK) homology domain. Here it colocalizes with E-cadherin and binds the APC tumour suppressor, which in turn regulates β-catenin. The interaction between hDlg and APC is via PDZ domain 2 and this is the same domain targeted by the high-risk HPV E6 proteins. Also shown is the interaction with protein 4.1, which connects hDlg to the actin cytoskeleton.

connected with both the actin cytoskeleton and the plasma membrane through binding to the cytoskeletal protein 4.1 (Lue et al., 1994; Marfatia et al., 1996) and with two members of the ERM family of cytoskeletal proteins, which in turn form a complex with the membrane glycoprotein CD44 (Lue et al., 1996). Moreover hDlg interacts with the MAGUK protein hCASK (Nix et al., 2000; Lee et al., 2002), which is also involved in connecting the actin cytoskeleton with the membrane junctions and the extracellular matrix through binding the 4.1 protein, the junctional adhesion molecule (JAM) and the syndecan-2 proteoglycan (Cohen et al., 1998; Martinez-Estrada et al., 2001). In addition, the PDZ domains of hDlg mediate interactions with the C-termini of several proteins including Shaker-type K^+ channels (Kim et al., 1995), and the APC tumour suppressor protein (Matsumine et al., 1996), which is mutated in the majority of colon cancers (Kinzler and Vogelstein, 1996). Indeed, complex formation between hDlg and APC was reported to block cell cycle progression (Ishidate et al., 2000). Recent observations have in fact pointed out that separate pools of Dlg protein may exist within the cell, which play different functions. While some specific hDlg isoforms are associated with the cell membrane at adherens junctions, others show nuclear localisation, possibly performing signalling functions (McLaughlin et al., 2002). Therefore, similarly to its *Drosophila* homolog, human hDlg protein appears to have different roles in governing both polarity and proliferation of epithelial cells.

High-risk HPV-16 and HPV-18 E6 proteins have been shown to bind to the PDZ domains of Dlg by means of their C-terminal PDZ-binding motifs (Lee et al., 1997; Kiyono et al., 1997), and also to target Dlg for ubiquitin mediated degradation (Gardiol et al., 1999; Kühne et al., 2000). It is possible that this occurs by enhancing a physiological process, since Dlg appears to be ubiquitinated and degraded by the proteasome in cells even in the absence of E6 (Gardiol et al., 1999; this thesis). The interaction between E6 and hDlg might be necessary at a defined point during the viral life cycle, in order to disrupt cell junctions and to abolish cell polarity, thereby altering the normal maturation and allowing proliferation of the infected keratinocytes. Alternatively, E6 might be targeting a different function of Dlg such as

growth inhibition. However, the consequences of Dlg degradation for the metastatic potential of tumours harbouring high-risk HPVs are dramatic. The C-terminal sequences of the E6 proteins of HPV-18 and HPV-16 which define a PDZ-binding motif differ at the last position, i.e. the sequence is ETQV in HPV-18 E6, and ETQL in HPV-16 E6. This single amino acid difference is sufficient to affect the binding affinities of these two proteins for their target PDZ domains on Dlg and MAGI-1 (Thomas et al., 2001). Consequently, HPV-18 E6 binds hDlg with higher affinity than HPV-16 E6 (Kiyono et al., 1997; Pim et al., 2000) and can degrade it more efficiently (Gardiol et al., 1999; Pim et al., 2000). Interestingly, this also correlates with the different malignant potential of the virus types, since it has been reported that cervical tumours associated with HPV-18 are more invasive and recurrent than those caused by HPV-16 (Burnett et al., 1992; Kurman et al., 1988; Zhang et al., 1995). This is in marked contrast with the reported lower efficiency of HPV-18 E6 in degrading p53 (Scheffner et al., 1990). Interestingly, low-risk HPV E6 proteins lack PDZ-binding motifs, and indeed they can neither bind hDlg (Kiyono et al., 1997; Lee et al., 1997) nor induce its degradation (Gardiol et al., 1999; Pim et al., 2000), however they acquire this ability if provided with a C-terminal PDZ binding domain derived from a high-risk E6 protein (Pim et al., 2000). In contrast, cutaneous HPV E6 proteins are unable to degrade hDlg even when the interaction is allowed by adding a PDZ-binding motif to their C-termini (D. Pim personal communication). This suggests that the mucosal HPV E6 proteins, whether derived from high or low-risk HPV types, can interact similarly with the cellular proteolytic machinery, whereas the high-risk cutaneous HPV E6 proteins would appear to significantly differ in this aspect.

Tight junctions (TJs) constitute the epithelial and endothelial junctional complex together with adherens junctions and desmosomes and are located at the most apical part of the complex. TJs have dual barrier and fence roles. They create the primary barrier to the diffusion of solutes through the paracellular pathway and maintain cell polarity as a boundary between the apical and basolateral plasma membrane domains (Gumbiner, 1993). It has been reported that expression of high-risk HPV E6 in mammalian cells is able to disrupt the integrity of epithelial tight junctions, and a

functional PDZ-binding motif was shown to be essential for this function of E6 (Nakagawa and Huibregtse, 2000). This strongly suggests that combined inactivation of different PDZ proteins by E6 might be responsible for the loss of epithelial cell adhesion and polarity of HPV-associated cancers. Indeed, during the last few years there has been an increasing number of reports on PDZ-containing proteins being targeted for ubiquitin-mediated degradation by the high-risk HPV E6 proteins. Among them hScrib, MAGI-1 and MUPP1 are all expressed at epithelial tight junctions, while hDlg is localised at adherens junctions.

hScrib is the human homologue of the *Drosophila* tumour suppressor Scrib, which cooperates with two other membrane-associated proteins, Dlg and Lgl, to control both formation of cell junctions and inhibition of epithelial cell growth, possibly by determining the localisation of growth factor receptors and signalling molecules (Bilder and Perrimon, 2000; Bilder et al., 2000). Human hScrib contains four PDZ domains and participates in the organisation of the epithelial tight junctions. hScrib has recently been shown to be a substrate for ubiquitination by the E6-E6AP complex *in vitro*, and for proteasome degradation mediated by high-risk HPV E6 *in vivo* (Nakagawa and Huibregtse, 2000).

E6 proteins from high-risk, but not low-risk HPV types were shown to bind another tight junction protein, MAGI-1 (Dobrosotskaya et al., 1997; Ide et al., 1999), through the first of its five PDZ domains (Thomas et al., 2001), and to reduce its steady-state levels and half-life (Glausinger et al., 2000). MAGI-1 forms a complex with β–catenin (Dobrosotskaya and James, 2000), whose expression is deregulated in many human cancers (reviewed in Polakis, 1999). Interestingly, the closely related proteins MAGI-2 and MAGI-3 are involved in the regulation of the PTEN tumour suppressor, a component of the Akt kinase signaling pathway that promotes cell survival and proliferation (Marte and Downward, 1997). MAGI proteins are required for enhancing the ability of PTEN to suppress Akt activation, probably through the assembly of a multiprotein complex at the cell membrane (Wu X et al., 2000; Wu Y et al., 2000). It is therefore plausible that MAGI-2/3 degradation, promoted by high-risk

HPV E6 (Thomas et al., 2002), might also affect Akt signalling and thereby inhibit apoptosis independently of targeting p53.

MUPP1 (Multi-PDZ domain-Protein-1) is a large multi-PDZ protein (Ullmer et al., 1998), which is concentrated at tight junctions of polarised epithelial cells. This probably occurs through its interaction with two integral membrane proteins that are basic constituents of TJ, claudin-1 and junctional adhesion molecule (JAM), which bind to adjacent PDZ domains of MUPP1 (Hamazaki et al., 2002). These features of MUPP1 are very similar to those of another tight-junction PDZ-protein: ZO-1; thus, similarly to what reported for ZO-1, MUPP1 could function as a crosslinker between claudin-based TJ strands and JAM oligomers within tight junctions. However, the most characteristic feature of MUPP1, i.e. the most characteristic difference of MUPP1 from ZO-1, is the occurrence of 13 PDZ domains in tandem within single MUPP1 molecules. This would indicate that many integral membrane molecules other than claudins and JAM can be tethered to the claudin-based TJ strands through MUPP1 molecules. Indeed, MUPP1 binds selectively, through its 10^{th} PDZ domain, to the cytoplasmic portion of the 5-HT_{2c} serotonin receptor (Ullmer et al., 1998; Becamel et al., 2001), and to the unphosphorylated c-Kit tyrosin kinase receptor (Mancini et al., 2000). Moreover, the membrane-spanning proteoglycan NG2 (Barritt et al., 2000) was also reported to bind MUPP1, although it remains unclear whether these membrane proteins are concentrated at TJs in epithelial cells. In addition to membrane proteins, MUPP1 would also recruit various cytoplasmic proteins to TJs. For example, it has recently been reported that the 10^{th} and 13^{th} PDZ domains of MUPP1 are a binding site for the TAPP1 protein (Kimber et al., 2002). TAPP1 interacts with PtdIns (3,4)P(2), a second messenger of growth factor- and insulin-induced signalling pathways, whose accumulation within the cell stimulates the translocation of TAPP1 to the plasma membrane where it binds MUPP1. Therefore MUPP1 has the capacity to assemble a large array of cellular proteins into a multitude of different signalling complexes beneath the plasma membrane at TJs. These protein clusters might play important roles in signal transduction pathways regulating the growth and/or differentiation of epithelial cells. We have previously shown that the Adenovirus type

9 transforming protein E4ORF1 (9ORF1) selectively binds MUPP1 PDZ domain 10 through its C-terminal PDZ-binding motif, and that functionally inactivates it through sequestration in cytoplasmic aggregates (Lee S. et al., 2000). In this thesis it will be shown how also HPV-18 E6 interacts with MUPP1 and reduces its protein stability through proteasome mediated degradation. Destruction of MUPP1 by E6 could thereby interfere with the assembly of signalling complexes at the epithelial tight junctions. Considering that two unrelated viral transforming proteins such as high-risk HPV E6 and Adenovirus 9 E4ORF1 appear to have both evolved to target the MUPP1 protein, is in favour of a role for MUPP1 in regulating crucial aspects of cell biology such as proliferation and/or maintenance of cell polarity. In contrast, HPV-18 E6 does neither interact with nor degrade the related TJ PDZ-proteins ZO-1 and ZO-2 (Lee S. et al., 2000; Glausinger et al., 2000).

It has been frequently reported that different oncogenic viruses inactivate the same cellular targets to overcome common obstacles to their replication: thus, there are viral oncoproteins other than HPV E6 which can bind PDZ-proteins, and this contributes to their transforming potential. hDlg has been shown to interact, through its PDZ domains, with both Adenovirus 9 E4ORF1 and HTLV-1 Tax, and these interactions interfere with the binding of APC, thereby perturbing cell growth control (Lee et al, 1997; Suzuki et al., 1999). Also MAGI-1 is bound by the Ad9 transforming protein E4-ORF1, which abolishes its activities by sequestrating it into cytoplasmic bodies similarly to what shown for MUPP1 (Glausinger et al., 2000). However, ubiquitin mediated degradation of PDZ-proteins is, to date, an exclusive property of the high-risk HPV E6 proteins. It has recently been shown that E6 can bridge the interaction between hScrib and the E6AP ubiquitin ligase, which normally would not recognise hScrib (Nakagawa and Huibregtse, 2000). This is reminiscent of p53 degradation, however a clear role for E6AP in the degradation of hScrib *in vivo* has not yet been confirmed. In contrast, E6 mediated proteasome degradation of hDlg would appear to be independent of E6AP (Pim et al., 2000), as deduced on the basis of E6's ability to degrade hDlg in extracts lacking E6AP, and on the fact that E6 mutants defective for E6AP binding and p53 degradation can still degrade hDlg. Similarly, low-risk E6

proteins can also target Dlg for degradation when provided with a PDZ-binding motif, however, these E6 proteins do not show any significant interaction with E6AP (Huibregtse et al., 1991; Huibregtse et al., 1993b). Moreover, no putative E6AP-binding site can be recognised within the Dlg PDZ domain bound by E6. However, the existence of a parallel pathway of Dlg degradation by E6 based on E6AP cannot be definitely excluded until E6AP-null cell lines are available.

E6 binding motifs.

It is clear from the above discussion that E6 is a multifunctional protein, which efficiently interferes with diverse cellular pathways. However, it is logical to ask how such a small and low abundant viral protein could evolve to interact with so many different cellular proteins, both with respect to the specificity of the interactions and to the relative abundance of the partners. The analysis of E6 targets has identified conserved E6 binding motifs, thereby characterising clusters of proteins, which are bound by E6 through similar domains. The PDZ domain, for instance, defines a family of proteins with common functions, which are targeted by high-risk E6 proteins through their C-terminal PDZ-binding motifs. E6 interaction with proteins containing multiple PDZ domains is, however, highly specific: thus, despite their high overall homology, only single PDZ domains on hDlg and MAGI-1 (Kiyono et al., 1997; Thomas et al., 2001) are recognised by the E6 protein. Another conserved motif, a charged leucine peptide has been found on a number of cellular targets of E6, including E6AP, hMcm7, E6BP and paxillin (Elston et al., 1998; Chen et al., 1998; Kühne et al., 1998). The binding specificity of E6AP for hMcm7 is mediated by this motif, present on both enzyme and substrate, and this in turn is used by E6 to interact with both (Kühne et al., 1998). Not surprisingly, E6 has also been reported to induce self-ubiquitination of E6AP (Kao et al., 2000), nonetheless, there are proteins which interact with E6 through this motif without being targeted for degradation, and these include E6BP (Chen et al., 1995) and paxillin (Tong and Howley, 1997). Interestingly,

inhibition of the charged leucine motif of BPV E6 resulted in repression of its transforming activity (Bohl et al., 2000).

The amount of E6 protein produced during a viral infection is very scarce, and the problem of targeting cellular proteins which are much more abundant within the cell is efficiently solved by inducing their ubiquitin-mediated degradation. Moreover, it seems plausible that the virus does not require the complete destruction of its targets, rather, even a transient decrease of their local concentration within the cell is likely to perturb the physiological conditions in favour of viral replication. This also implies that E6 does not need to interact with all of its putative targets at the same time, since probably only a limited subset will be available at defined stages during cell differentiation and in specific compartments within the cell. Indeed, different studies have reported E6 to be localised either in the nucleus, cytoplasm or membranes (Androphy et al., 1985; Liang et al., 1993; Sherman and Schlegel, 1996). It is therefore possible that the intracellular localisation of E6 might change, either as a consequence of differentiation or as a result of exogenous stimuli. Indeed, the PDZ-binding specificity of high-risk E6 has also been shown to be regulated by a cellular pathway. The E6/hDlg interaction is inhibited by PKA phosphorylation of a conserved threonine residue within the PDZ binding domain on E6, and this was shown to inhibit also E6-mediated degradation of hDlg. Indeed, high levels of hDlg could be restored in HPV-18 positive cervical cancer cells by induction of PKA activity (Kühne et al., 2000). Interestingly, PKA regulation of PDZ domain binding was also reported to control the interaction between the PDZ protein PSD-95 with both the K^+ channel Kir2.3 (Cohen et al., 1996) and the glutamate receptor binding protein stargazin (Choi et al., 2002). This would suggest that the high-risk HPV E6 proteins might have acquired the PDZ binding domain, with its PKA-associated regulation, from the cellular genome. Its strict conservation among all high-risk E6 proteins is intriguing, and implies a requirement for the virus to finely balance its effects upon hDlg and other PDZ domain containing proteins. Recent studies have also shown that E6 is phosphorylated by PKN (Gao et al., 2000), and it would be interesting to evaluate whether this also regulates any of E6's other activities.

Objectives of the study.

From the above introduction it is clear that E6 is a multifunctional protein whose activities are not simply required to counteract the apoptotic response to E7-induced proliferation, rather to play on several fronts within the infected cell in order to create a favourable environment for viral replication. The aim of this thesis is to investigate how E6 impinges upon diverse cellular pathways, whose inactivation appears central to tumour progression.

Firstly the activation of the p53 tumour suppressor will be examined in HPV-positive cancer cells when E6-mediated degradation is blocked. In this way it will be possible to study whether E6 interferes with the functions of p53 independently of targeting it for degradation. In addition, the activity of HPV E6 towards the multi-PDZ tight junction protein MUPP-1 will be analysed. Finally, since high-risk HPV E6 has been demonstrated to target the MAGUK protein Dlg for degradation, the cellular mechanisms which regulate the stability of this tumour suppressor will be studied in both HPV-positive and negative epithelial cells. In particular, attention will be dedicated to the consequences of the loss of Dlg regulation during the metastatic stages of tumour progression.

These studies will be performed in the light of identifying new possible targets for therapeutic intervention against HPV-associated cervical cancer.

RESULTS AND DISCUSSION

PART 1.

Inhibition of proteasome-mediated degradation in HPV-positive cancer cells is not sufficient for accumulation of p53 protein.

In contrast to many other tumours, p53 mutations are very rarely detected in HPV-associated cervical cancers (Fujita et al., 1992; Park D. et al., 1994), since its functions are antagonised by the high-risk HPV E6 oncoprotein. However, the presence of high-risk HPV sequences does not appear to be functionally equivalent to inactivating mutations of the p53 gene. In fact it has been shown that, although p53 protein levels are invariably quite low in cell lines derived from cervical tumours, consistent with the notion of HPV E6-mediated degradation, the pathways leading to induction of p53 are nonetheless intact. Thus, treatment with certain DNA damaging agents has been found to inhibit the expression of the HPV E6/E7 oncogenes (Butz et al., 1996), often resulting in increased p53 protein levels and, in some cases, in an increase of p53 transcriptional activity (Butz et al., 1995; Butz et al., 1999). This implies that the downstream pathways induced by activated p53 are still functional in these cervical tumour derived cell lines. Indeed, in some cases targeting the expression of the E6/E7 oncogenes, also results in growth inhibition of tumour cells following p53 accumulation (von Knebel-Doeberitz et al., 1988, 1992; Butz et al., 1996; Alvarez-Salas et al., 1998).

An important question arising from these data is whether inhibition of E6-mediated degradation of p53 in HPV-positive tumour cells will result in an activation of p53 without concomitant stimulation by genotoxic agents. This is particularly important from a therapeutic point of view, since it will determine whether the appropriate signals are already present within these cells to activate p53.

Comparison of the efficacy of different proteasome inhibitors in stabilising p53 protein.

Since p53 is targeted for degradation by E6 through the ubiquitin-proteasome pathway (Scheffner et al., 1990), an obvious means of addressing the question of whether p53 is intrinsically activated in cancer cells expressing high-risk HPV E6, is to monitor p53 protein levels following proteasome inhibition. A number of proteasome inhibitors of varying specificity have been described (Rock et al., 1994, Fenteany et al., 1998; Lee et al., 1998), therefore the effects of lactacystin (LC) and N-acetyl-leu-leu-norleucinal (LL) were first determined upon p53 protein levels in HPV-16 (CaSKi) and HPV-18 (HeLa) containing cervical tumour derived cell lines. Cells were treated for two hours with the proteasome inhibitors, either in the presence or absence of the DNA damaging agent mitomycin C for 18 hours. Subsequently, the cells were harvested and p53 levels determined by Western blot analysis with a pool of anti-p53 monoclonal antibodies. The results obtained are shown in Figure 7. As can be seen, treatment of CaSKi cells with either LC or LL results in similar levels of induction of p53 protein. Interestingly, the slower migrating form of p53, corresponding to the p53 Pro variant is protected to a greater extent and this is consistent with previous observations (Storey et al., 1998). Treatment with mitomycin C alone results in no significant increase in p53 levels. In marked contrast, addition of either LC or LL to the HeLa cells has very little effect on the steady state level of p53 within these cells. Even mitomycin C treatment induces only a modest increase in p53 levels, and this is in agreement with previous results (Butz et al., 1995). It should be noted that this increase could be, at least in part, a consequence of the reported downregulation of E6 expression levels induced by mitomycin C treatment (Butz et al., 1996). However, in both HeLa and CaSKi cells, p53 is most readily detected following DNA damage induction if the proteasome inhibitor LL is also present. This suggests that, in contrast to the physiological Mdm2-dependent degradation of p53, E6-E6AP mediated degradation of p53 is not inhibited by DNA damage. However, the most striking conclusion that can be obtained from these results is that inhibition of E6 mediated

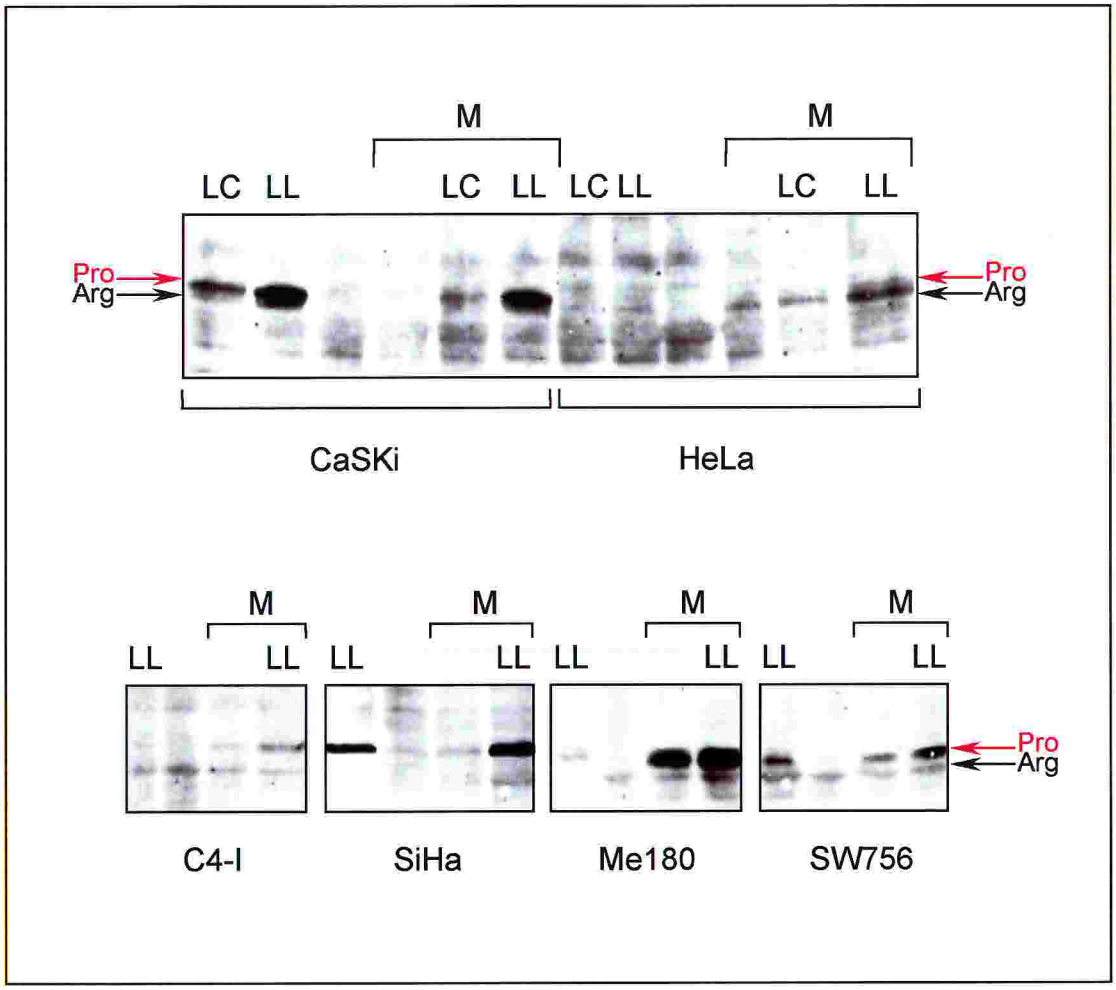


Figure 7. Inhibition of E6 mediated degradation of p53 in HPV-positive cell lines.

HPV-16 positive CaSKi and SiHa cells, HPV-18 positive HeLa, C4-I and SW756 cells, plus HPV-68 positive Me180 cells were either left untreated or treated with mitomycin C (M) for 18 hours, and with proteasome inhibitors lactacystin (LC) or N-acetyl-leu-leu-norleucinal (LL) for two hours before harvesting. p53 levels were then assessed by Western blotting. Pro and Arg polymorphic forms of p53 are indicated by arrows. Background staining confirms equal levels of protein loading.

degradation in CaSKi cells will result in a significant upregulation of the p53 protein levels, whereas this is not the case in HeLa cells. Additional stimulation by induction of DNA damage is required before a significant accumulation of p53 protein can be obtained in HeLa cells.

Differential induction of p53 in HPV-positive cells following inhibition of proteasome-mediated degradation.

Having shown that differences exist in the induction of p53 protein between the CaSKi and HeLa cell lines, it was then interesting to examine the effects of proteasome inhibition upon p53 levels in a variety of other HPV DNA-containing cervical tumour derived cell lines. Since the highest level of p53 protection was obtained using LL as opposed to LC, the remaining studies were performed using LL. The induction of p53 protein was compared in four different cell lines derived from cervical tumours: C4-I (HPV-18), SiHa (HPV-16), Me180 (HPV-68) and SW756 (HPV-18). The cells were treated with LL plus or minus mitomycin C, and the p53 protein levels were ascertained as described above. From the results shown in Figure 7, it is clear that the pattern of p53 protection-induction seen in the SW756 and SiHa cells is similar to that seen in the CaSKi cells. Inhibition of E6-mediated degradation of p53 gives rise to a dramatic increase in p53 protein levels, which is consistent with there being signals already present within these cells for activating and stabilising the p53 protein. However, proteasome-mediated degradation of p53 appears to occur at a very high rate in these cell lines, as indicated by the fact that stimulation with mitomycin C alone only gives rise to a weak increase in the p53 levels, which is further augmented by concomitant proteasome inhibition.

In contrast, the C4-I and, to a slightly lesser extent, the Me180 cells both behave like the HeLa cells. Proteasome inhibition does not result in a significant increase in the p53 levels, indicating that there is no intrinsic activation of p53 within these cells. Only following treatment with a DNA damaging agent is a significant increase in the

levels of p53 protein detected, and proteasome inhibition leads to further accumulation of p53 protein.

For comparison, the effects of proteasome inhibition on p53 levels were then examined in two cell lines lacking HPV E6 sequences. HT1080 cells contain wild type p53 protein and, as can be seen from Figure 8A, proteasome inhibition gives rise to a modest increase in p53 levels, consistent with the observation that p53 is normally regulated via the proteasome pathway (Kubbutat et al., 1997; Haupt et al., 1997). DNA damage induction results in a dramatic increase in the p53 levels and, in contrast to the HPV E6 containing cell lines, this is not augmented by treatment with proteasome inhibitors, consistent with the notion that DNA damage induction shuts off degradation of p53 in cells lacking HPV E6. In contrast in C33-I cells, which have mutant p53 protein (Scheffner et al., 1991), the levels of p53 are completely unaffected by either DNA damage or treatment with proteasome inhibitors.

Having demonstrated differences in the intrinsic activation of p53 in cell lines derived from cervical tumours it was then interesting to investigate the effects of proteasome inhibition in cell lines immortalised by HPV-16 but which are not fully transformed. To do this W12, V1/G and AC89/E2 keratinocytes were analysed as described above, and the results obtained are shown in Figure 8B. In the case of V1/G cells no detectable p53 protein was found. However, in both the AC89/E2 and W12 cells a strong induction of p53 protein was obtained following proteasome inhibition. These results indicate that, at least in the case of these two immortalised keratinocyte lines, the intrinsic activation pathways to p53 are active in the absence of any additional stimulus.

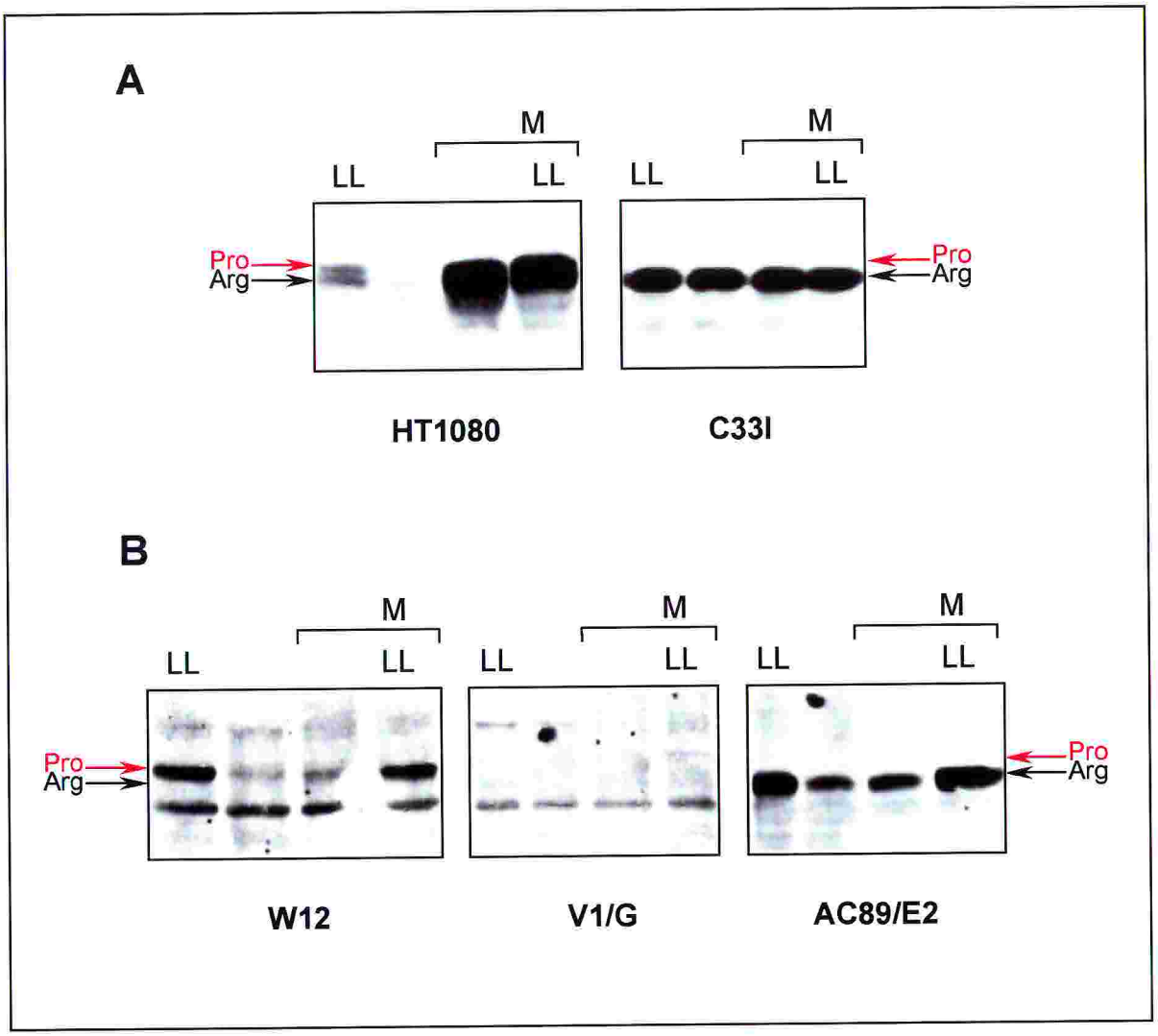


Figure 8. Effects of DNA damage and proteasome inhibition on p53 induction in different epithelial cell lines.

(A) HPV-negative HT1080 human fibrosarcoma cells and C33I cervical carcinoma cells, and **(B)** HPV-16 immortalised keratinocyte cell lines W12, V1/G and AC89/E2 were either left untreated or treated with mitomycin C (M) for 18 hours and with N-acetyl-leu-leu-norleucinal (LL) for two hours before analysing p53 levels by Western blotting. The Pro and Arg polymorphic forms of p53 are indicated by arrows.

The Pro/Arg polymorphism at position 72 of p53 is not responsible for differences in the intrinsic activation of p53.

Since previous work from our group had demonstrated that the p53 polymorphism at position 72 can affect the susceptibility of p53 to E6 mediated degradation (Storey et al., 1998), we were next interested in investigating whether particular polymorphic forms of p53 were more likely to be intrinsically activated than others in HPV-associated cervical cancers. To do this the polymorphic status of p53 was determined in all the cell lines analysed, by amplifying a fragment of the p53 mRNA containing the polymorphic codon through RT-PCR and subsequently sequencing the PCR products. A representative sequence analysis is shown in Figure 9 and the results are summarised in Table 3. As can be seen, in the nine HPV-containing cell lines analysed there was a broad spectrum of homozygous p53 Arg, homozygous p53 Pro and heterozygous p53 containing cell lines. Of those cell lines which gave rise to the weakest induction of p53 following proteasome inhibition, C4-I were homozygous p53 Pro and both HeLa and Me180 were heterozygous, although the level of detection of the Pro allele in HeLa cells was consistently low, the significance of which remains to be determined. However, based on this analysis, the polymorphic status of p53 within these lines does not appear to be responsible for differences in the levels of intrinsic activation in the absence of additional stimuli.

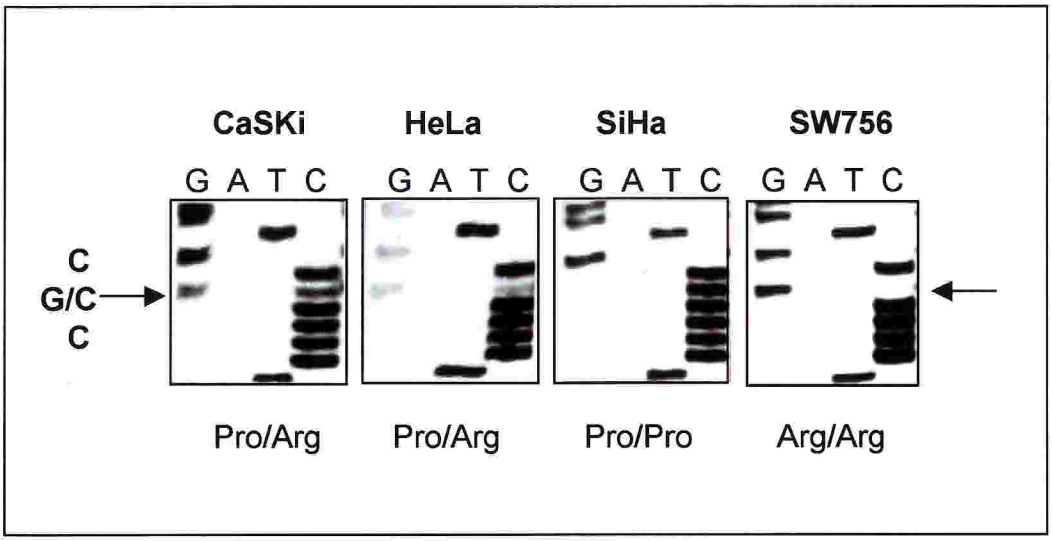


Figure 9. Sequence analysis of the Pro/Arg polymorphic status of p53 at codon 72 in different HPV DNA-containing cell lines.

The p53 cDNA region comprising codon 72 was amplified by RT-PCR from total RNA of different cell lines and subsequently analysed by cycle sequencing. Arrows indicate the polymorphic base at second position of codon 72: CCC=Pro, CGC=Arg. Cell lines heterozygote for the Pro and Arg alleles have both C and G at this position.

Cell line	HPV type	P53 Pro/Arg status
CaSKi	16	Pro/Arg
SiHa	16	Pro
W12	16	Pro
Ac89/E2	16	Arg
V1/G	16	Arg
HeLa	18	Pro/Arg
C4-I	18	Pro
Me180	68	Pro/Arg
SW756	18	Arg
HT1080	-	Pro/Arg
C33I	-	Arg

Table 3. Polymorphic status of the p53 gene and HPV type in the cell lines analysed.

Nuclear localisation of p53 is perturbed in cancer cells expressing HPV E6.

Previous studies have shown that in normal cells, activation of p53 results in its nuclear accumulation and induction of a variety of p53 responsive promoters (el-Deiry et al., 1993; Pietenpol et al., 1994; Crook et al., 1998). We were next interested in determining whether p53 can efficiently localise to the nucleus when HPV E6 is present, particularly in those cell lines in which DNA damage and proteasome inhibition can induce high levels of p53. To address this question, a series of immunofluorescence assays were performed on HT1080 cells, which contain wild-type p53 and lack HPV E6 protein, on Me180 cells which contain wild-type p53 and harbour high-risk HPV-68 E6, and on SiHa cells which harbour wild-type p53 and high-risk HPV-16 E6. Cells were treated with LL for two hours in the presence or absence of mitomycin C, and were then fixed and probed for p53 protein. As can be seen in Figure 10, treatment of HT1080 cells with LL results in a weak accumulation of p53 within the nucleus, consistent with the Western blot analysis shown above (Figure 8A). Induction of DNA damage with mitomycin C gives rise to a strong nuclear localisation of p53, and a similar effect is seen when LL is also added to the cells. In contrast, the treatment of Me180 cells with mitomycin C produces a marked increase in perinuclear staining, but a very limited accumulation within the nucleus can be detected in comparison with that seen in the HPV-negative HT1080 cells (Figure 11A). Even following proteasome inhibition there is a dramatic increase in the levels of p53 protein, but very little nuclear localisation is detected within the time frame of this assay. In the SiHa cells, where accordingly to the Western blot analysis p53 appears to be intrinsically activated, it also fails to localise efficiently to the nucleus, even after DNA damage and proteasome inhibition (Figure 11B). These results demonstrate that even if ubiquitin mediated degradation of p53 is blocked, its localisation to the nucleus is strongly inhibited in cells containing HPV E6.

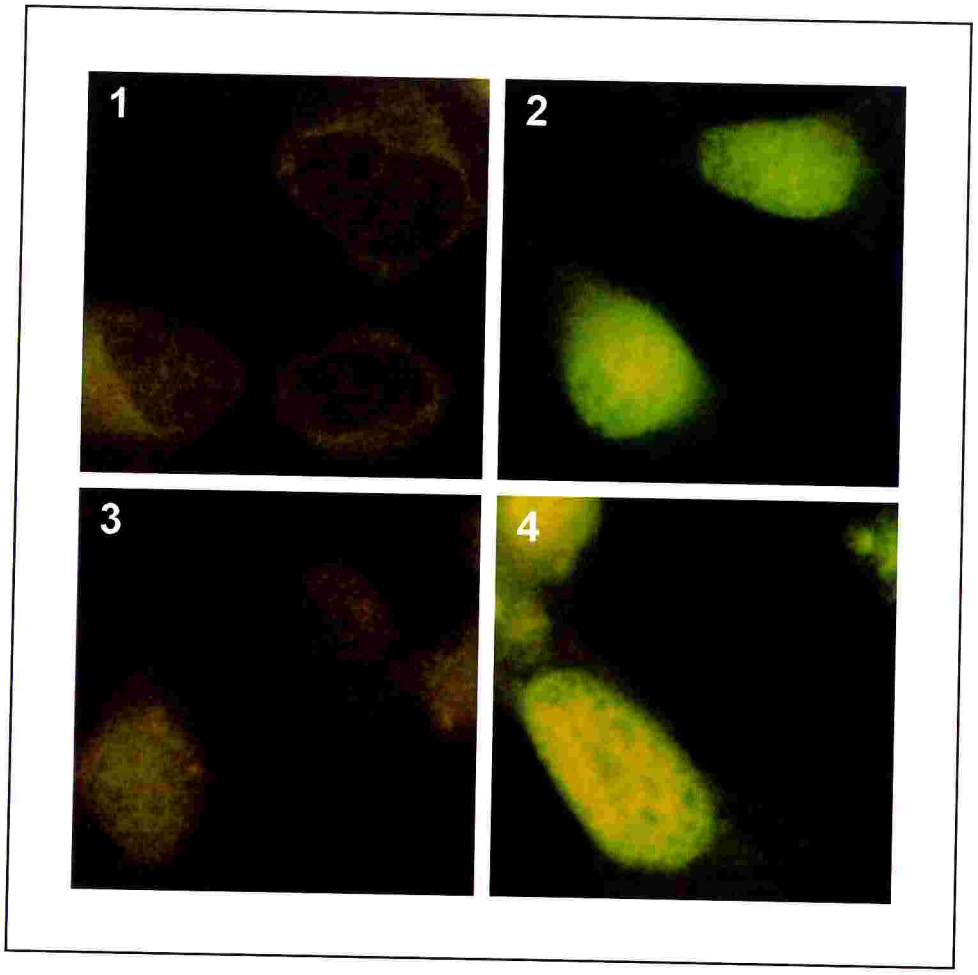


Figure 10. Activated p53 localises to the nucleus of HPV-negative HT1080 cells.

HT1080 human fibrosarcoma cells were either:
1) untreated
2) treated with mitomycin C for 18 hours
3) treated with LL for 2 hours
4) treated with both mitomycin C and LL
p53 localisation was then checked by immunofluorescence analysis.

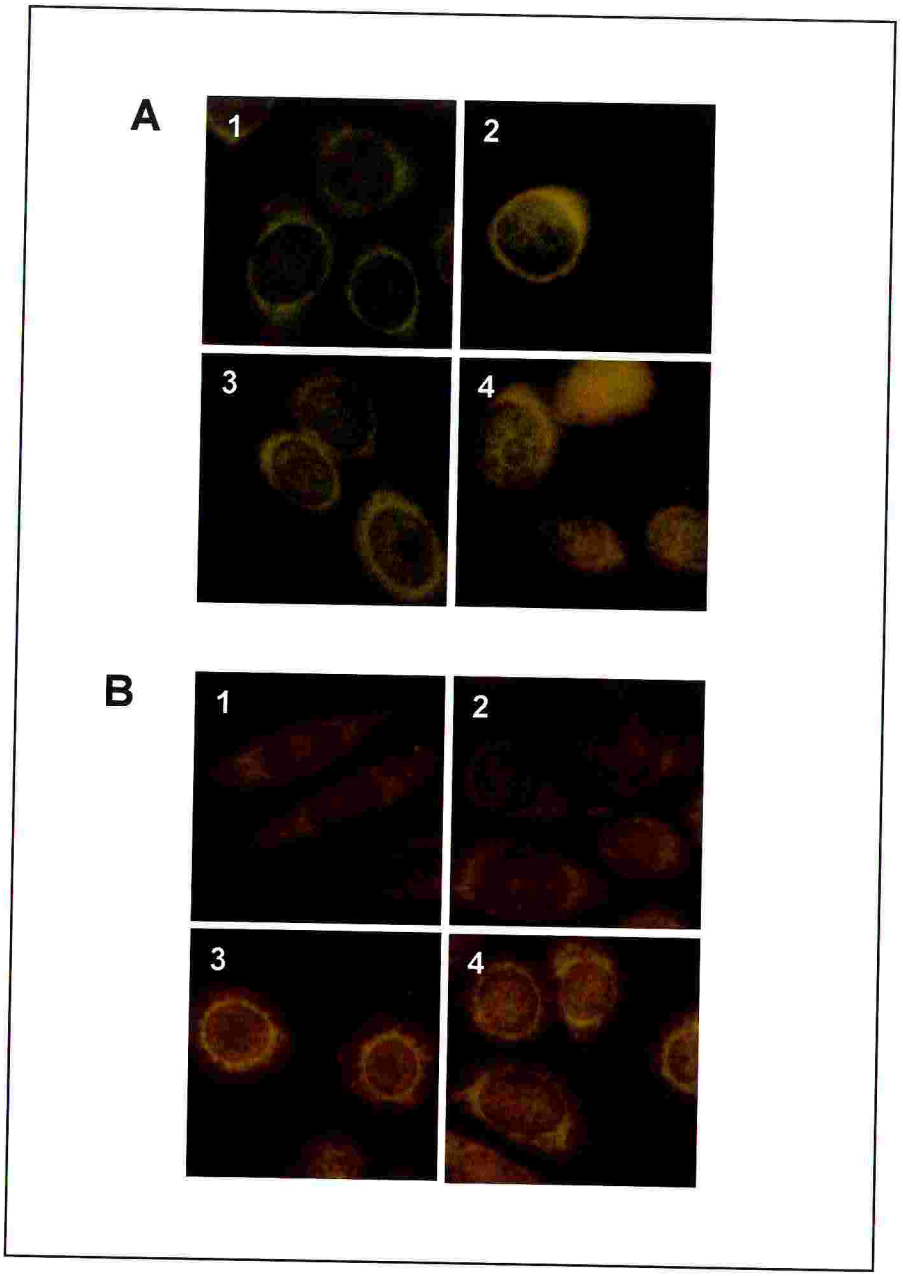


Figure 11. p53 nuclear localisation is perturbed in HPV-positive cancer cells.

HPV-68 positive Me-180 cells **(A)** and HPV-16 positive SiHa cervical cancer cells **(B)** were either:

1) untreated

2) treated with mitomycin C for 18 hours

3) treated with LL for 2 hours

4) treated with both mitomycin C and LL

p53 localisation was then determined by immunofluorescence analysis.

DISCUSSION

Since HPV E6 is invariably expressed in cervical lesions, and it would also appear to be responsible for their malignant progression, this protein represents an attractive candidate for developing therapeutic strategies directed against cervical cancer. Moreover, a therapy directed towards a noncellular factor should allow a specific attack on HPV-positive cells. Diverse approaches have been tested in order to block the expression of E6 in HPV-positive cervical cancer cells: e.g. selective inhibition of viral transcription (Goodwin and DiMaio, 2000), transfection of antisense constructs (Hamada et al., 1996; Hu et al., 1995; von Knebel Doeberitz et al., 1992) or ribozymes directed against the polycistronic E6/E7 mRNA (Alvarez-Salas et al., 1998). All these attempts have been reported to cause growth suppression and concomitant reduction of tumorigenicity *in vivo*, implying the feasibility of reactivating functional tumour suppressor pathways in HPV-positive cells. However all these strategies have the drawback of concomitantly repressing both the HPV E6 and E7 oncogenes, that are expressed together from a polycistronic transcript. In this way, the proapoptotic stimulus exerted by E7 is lost, and the therapy can only result in growth suppression of cancer cells, which however implies the need of continuously administrating the therapeutic agents. Since HPV E6 has been frequently reported to have a strong anti-apoptotic effect in HPV-positive cancer cells, it could be reasoned that interfering with the activities of E6, while retaining those of E7 as a proapoptotic stimulus, may provide a higher therapeutic potential. Peptide aptamers have been used to block HPV-16 E6 activities *in vivo*, and their administration was indeed found to induce apoptotic death of HPV-16 positive cancer cells (Butz et al., 2000). It is widely assumed that E6 mainly exerts its antiapoptotic activity by targeting the p53 tumour suppressor for ubiquitin-mediated degradation (see introduction). Therefore, it can be reasoned that the synthesis of specific inhibitors of the E6/E6AP/p53 complex might provide a valid therapeutic approach against HPV-induced cancers. In general, however, strategies aimed at blocking specific interactions between E6 and its cellular targets are difficult. This is due to the nature of the E6-target protein interactions,

which are frequently overlapping and span large regions of the E6 protein. Thus attempts to eliminate one set of interactions are very likely to also perturb others. Moreover, an additional note of caution is required, since targeting the p53/E6AP/E6 complex could also interfere with normal E6AP functions, and mutations of E6AP have been implicated in a serious developmental disorder, the Angelman Syndrome (see above). Nonetheless, blocking the E6-mediated degradation of p53 is a major therapeutic goal, which still represents an attractive strategy to induce a p53-mediated apoptotic response in HPV-transformed cells. Indeed, it has been reported that the p53-responsive pathways are often functional in cervical tumour cell lines (Butz et al., 1995; Butz et al., 1999). This is an extremely important point, since blocking p53 degradation does not necessarily imply its activation as a transcription factor. For example, unmodified p53 stabilised by treatment with a proteasome inhibitor, shows the same kinetics of DNA binding as p53 from cells treated with ionising radiation (IR), however it does not have the same ability to transactivate downstream targets as p53 of IR-damaged cells (Siciliano et al., 1997).

In order to establish if such an approach would be applicable for a therapy directed against HPV-associated cancers, we decided to analyse whether intrinsic signals were present within HPV-containing tumour cell lines, which would stabilise and activate the p53 protein if E6-mediated degradation was inhibited. To address this question, proteasome inhibitors were assessed on a variety of tumour-derived and of HPV-immortalised cell lines. In most cases proteasome inhibition indeed resulted in increased steady state levels of p53 protein, suggesting that in these cases it was already activated within the cells. In three cases however, proteasome inhibition alone did not result in increased p53 protein levels, indicating that in these cell lines the signals required to activate p53 were missing. Only after the additional insult of treatment with a DNA damaging agent was the p53 protein induced.

In an attempt to search for mechanistic explanations to the failure of p53 induction, we determined the polymorphic status of p53 in all cell lines analysed, with respect to the Pro/Arg residue at position 72. Indeed, it had been previously shown that the Arg form of p53 is more susceptible to E6 mediated degradation (Storey et al., 1998).

However, our analysis did not highlight any correlation between the allelic status and the activation status of p53. Interestingly, looking at the type of HPV infection in the tumour cell lines analysed, it can be noted that the great majority of the HPV-16 positive cell lines (CaSKi, SiHa, W12 and AC89/E2) showed high levels of intrinsic activation of p53, as demonstrated by its efficient accumulation following inhibition of proteasome-mediated degradation. The only exception to this was represented by the V1/G keratinocytes, where no detectable levels of p53 protein could be seen even upon treatment with genotoxic agents. In contrast, of the cell lines lacking intrinsic p53 activation both HeLa and C4-I contain HPV-18 DNA, and the Me180 cells contain HPV-68 (Gravitt et al., 1998), which is highly homologous to HPV-18 (Myers et al., 1994). This finding is somewhat surprising, since HPV-18 E6 was reported to be less efficient than HPV-16 E6 in targeting p53 for degradation (Scheffner et al., 1990). However it is also well established that those tumours harbouring HPV-18 DNA are more aggressive and more prone to recurrence than those associated with HPV-16 (Burnett et al., 1992; Kurman et al., 1988; Zhang et al., 1995). Although the number of cell lines used in this study is small, it would be nonetheless interesting to analyse a statistically significant number of HPV-positive cancers in order to establish whether the lack of intrinsic activation of p53 might represent a general feature of HPV-18 positive tumours.

The failure to induce p53 upon treatment with proteasome inhibitors in some of our cell lines could be the consequence of mutations in genes that regulate the cellular pathways leading to p53 activation. It is well established that the p53 gene is almost invariably wild type in HPV-associated cancers, even in recurrent cervical tumours (Denk et al., 2001), although p53 mutations have been reported to occur in cervical tumours at the metastatic stage of progression (Crook and Vousden, 1992). An alternative explanation however, would be that the block to p53 induction is a direct consequence of the activities of the viral oncoproteins. For instance, it might be possible that the HPV-18 E7 oncoprotein is less effective than its HPV-16 counterpart in inducing p53 accumulation. It would then be tempting to speculate that for this reason the HPV-16 E6 protein had to evolve to degrade p53 with a higher efficiency

than HPV-18 E6. At this stage it is not possible to determine whether the lack of p53 induction in some cervical tumour cell lines is due to the activities of the viral oncoproteins or is the result of secondary mutations upstream of p53. However, a possible approach to try to resolve this issue would be to examine the effects of proteasome inhibitors in keratinocyte cell lines newly derived following transfection of the E6/E7 oncogenes from either HPV-16 or HPV-18.

Other points also needed to be considered in order to test the effects of blocking p53 degradation on its biological activity. In normal, unstressed cells, latent p53 is nuclear only from mid G1 to the G1/S transition of the cell cycle, and it becomes increasingly cytoplasmic as the cell progresses through the cell cycle (David-Pfeuty et al., 1996). Exposure of the cell to stress results in accumulation of activated p53 within the nucleus (Fritsche et al., 1993), where it exerts its function as a transcription factor on a variety of target promoters (el-Deiry et al., 1993; Pietenpol et al., 1994; Miyashita and Reed, 1995; Knippschild et al., 1996; Crook et al., 1998). A key question in the therapeutic reactivation of p53 in HPV positive tumour cells is whether or not this process of p53 nuclear relocalisation can efficiently take place in the presence of E6. To investigate this question we performed a series of studies on cells containing wild type p53, either with or without high-risk HPV E6. The accumulation and subcellular localisation of p53 were monitored following both proteasome inhibition and induction of DNA damage. The HPV-containing cell lines chosen for this analysis were Me180 and SiHa, which we have found to induce relatively high levels of p53 protein following proteasome inhibition and treatment with a DNA damaging agent. Strikingly, in comparison to the HT1080 cells, in which p53 was found to localise efficiently to the nucleus following proteasome inhibition and genotoxic insult, in both Me180 and SiHa cells p53 remained predominantly cytoplasmic. It should be mentioned at this point that the perinuclear accumulation of p53 seen in Me180 and SiHa cells upon treatment with mitomycin C was not due to the cells arresting in the G2 phase of the cell cycle, since we monitored the cell cycle profiles of the cell lines tested and did not detect any cell cycle blocks after either proteasome inhibition or genotoxic insult, and this is in accordance with previous reports (Butz et al., 1995). It

is also worth remembering that genotoxic treatment of HPV-positive cancer cells has been reported to cause repression of E6 and E7 oncogene expression (Butz et al., 1996), and this may account for a slight increase in p53 nuclear staining that could be seen in Me180 cells upon treatment with mitomycin C, but not in the cells treated with proteasome inhibitors alone.

These results demonstrate that even if p53 degradation by E6 is blocked by proteasome inhibition, the p53 protein fails to localise to the nucleus, and this suggests that complex formation between E6 and p53 is sufficient to perturb p53 nuclear localisation. This provides an explanation for previous studies which have shown that expression of high-risk HPV E6 inhibits p53-mediated activation *in vivo* independently of its ability to promote p53 degradation (Pim et al., 1994), since the failure of p53 to localise in the nucleus would certainly be sufficient to inhibit its transcriptional activity. Moreover, our observations are in agreement with previous results showing that a p53-responsive promoter element was scarcely activated following transient transfection in Me180 cells, and that its activation was much lower in SiHa cells (Butz et al., 1995). Consistently, it has also been reported that mitomycin C treatment of HPV-positive SiHa and HeLa cells does not lead to an induction of the p53-responsive gene p21/WAF1 in these cell lines (Butz et al., 1995). Moreover, a slight induction of the p53-responsive gene GADD45 has been observed in Me180 cells after mitomycin C administration, while in SiHa cells this was completely blocked (Butz et al., 1999). All these results correlate perfectly with our observations, since a small fraction of p53 can still localise to the nucleus of Me180 cells upon genotoxic treatment, whereas nuclear exclusion appears to be more dramatic in SiHa cells. It would certainly be interesting to check a more wide range of HPV-positive cells and monitor the efficiency in nuclear exclusion of p53.

The finding that HPV E6 can cause nuclear exclusion of p53 independently of its degradation is not surprising, since cytoplasmic sequestration of p53 is a common strategy adopted by different viral proteins. Adenovirus E1B 55K anchors p53 in cytoplasmic structures, reducing its transcriptional activity (König et al., 1999), and p53 has been found to be sequestrated in the cytoplasm of Cytomegalovirus-infected

human endothelial cells (Kovacs et al., 1996). The Hepatitis B virus HBV X protein concentrates p53 in the cytoplasm, inhibiting both its transactivation and proapoptotic functions (Elmore et al., 1997; Ueda et al., 1995). Hepatocellular carcinoma is among one of the most common malignancies, with over 80% of patients positive for Hepatitis B virus infection. As p53 mutations are found in only one-quarter to one-third of these tumours (Ueda et al., 1995, and references therein), cytoplasmic sequestration is clearly an important mechanism of inactivating p53. Moreover, many tumours have been reported to contain wild type p53, whose functions are however inactivated due to constitutive cytoplasmic sequestration (Moll et al., 1992; Bosari et al., 1995; Schlamp et al., 1997), and this is associated with tumour metastasis and poor long-term patient survival (Sun et al., 1992). It has been demonstrated that the carboxyl-terminal region of p53 protein is responsible for HBV X protein-dependent cytoplasmic localisation (Takada et al., 1997). Interestingly, this same region has been shown to contain a functional nuclear export signal (Stommel et al., 1999), therefore it could be hypothesised that HBV X protein acts by enhancing p53 nuclear export rather than inhibiting its nuclear import. Retention of p53 in the cytoplasm of HPV-positive cancer cells might be either due to E6 masking a nuclear localisation signal by binding to the p53 C-terminus, or to enhanced nuclear export of p53. Facilitated nuclear import of p53 occurs via three lysine-rich nuclear localisation signals at amino acids 305-322 (NLS I), 369-375 (NLS II) and 379-384 (NLS III) (Liang et al., 1998; Shaulsky et al., 1990). The E6 binding site on p53 C-terminus has been mapped to amino acid residues 356-380: this would be expected to block the NLS II and III but not NLS I, which appears to be the most active signal (Shaulsky et al., 1990). Moreover, the possibility that E6 may be retaining p53 in the cytoplasm through physical association seems unlikely given the low abundance of the E6 protein in the cell. The evidence supporting a role for E6 in inducing p53 nuclear export is more convincing. It has been observed that inhibition of nuclear export in HPV-positive tumour cells by the drug leptomycin B (LMB) results in stabilisation of p53 protein, indicating that E6-mediated degradation of p53 is, at least in part, dependent on nuclear export (Freedman and Levine, 1998). More recently it has been reported that

blocking the interaction between Mdm2 and p53 results in nuclear accumulation of p53 in HPV-negative, but not HPV-positive cells. In contrast, combined treatment of HPV-positive cervical cancer cells with the drugs LMB and Actinomycin D results in nuclear accumulation of p53. This is likely due to the synergistic action of Actinomycin D downregulating E6/E7 mRNA expression, and of LMB inhibiting nuclear export (Hietanen et al., 2000). Moreover, although these data strongly suggest that E6-induced degradation of p53 occurs in cytoplasmic proteasomes, colocalisation of E6 and p53 proteins in the cytoplasm is not alone sufficient to trigger degradation. Indeed, it has been observed that in neuroblastoma cells p53 is constitutively cytoplasmic and appears to be resistant to proteolysis induced by either Mdm2 or E6 (Isaacs et al., 1999), implying the requirement for nucleocytoplasmic shuttling in order to allow degradation. Whether this process is mediated directly by E6, its cellular partner E6AP, or another protein, however remains to be determined, since E6, E6AP and p53 all contain putative nuclear export signals. A series of recent reports have suggested that nuclear export of p53 is a direct consequence of its ubiquitination by Mdm2 (Lohrum et al., 2001; Gu et al., 2001), and indeed an intact RING finger on Mdm2 is required to promote nuclear export of p53 (Geyer et al., 2000; Boyd et al., 2000). It has been proposed that ubiquitination induces conformational changes, which reveal a nuclear export signal on p53. It seems likely that HPV E6 could also have a similar effect on p53, since it can induce ubiquitination of p53 in the nucleus (Vogt Sionov et al., 2001). Indeed c-Abl, which prevents the ubiquitination and nuclear export of p53 by Mdm2 in response to DNA damage, has been found to inhibit both ubiquitination of p53 by the E6-E6AP complex and its nuclear export in cells overexpressing E6 and in HeLa cells (Vogt Sionov et al., 2001). Interestingly, c-Abl does not appear to inhibit the binding between E6 and p53. Therefore it can be hypothesised that the nuclear exclusion of p53 seen in cervical cancer cells is a consequence of the E6-E6AP mediated ubiquitination of p53. The catalytic nature of this process could explain how E6 can achieve such an effect despite its low abundance in the cell. At present, our data cannot formally exclude the possibility that the nuclear exclusion of p53 is not due solely to the presence of E6, but rather may be

ascribed to the HPV E7 oncoprotein. Other experimental approaches would be required in order to address directly this possibility. However, it has been observed that in normal diploid human fibroblasts expressing HPV-16 E7 p53 is upregulated and its nuclear localisation is not altered (Seavey et al., 1999; Eichten et al., 2002), although it appears to be transcriptionally inactive (Eichten et al., 2002). Moreover, it has been recently shown that treatment of HPV-16 positive SiHa cells with a peptide aptamer which interacts specifically with HPV-16 E6 results in the accumulation of p53 protein within the nucleus, and in concomitant induction of apoptosis (Butz et al., 2000). These results are in favour of a direct role of the E6 protein in the nuclear export of p53.

In conclusion, the above results demonstrate that inhibition of E6 induced degradation of p53 in cell lines derived from cervical tumours frequently gives rise to an increase in the levels of p53 protein. However, cases exist where induction of p53 cannot be obtained without additional genotoxic insult. In other words, our observations would indicate that a therapeutic strategy aimed at blocking E6-mediated degradation of p53 is unlikely to be universally applicable. Moreover, even when its degradation is blocked, p53 fails to localise to the nucleus even if stimulated by additional genotoxic insult. Therefore, to increase the probabilities of achieving a successful therapeutic strategy, this should be directed more towards blocking the interaction between the E6 and p53 proteins, rather than simply interfering with E6-mediated proteolytic activity.

PART 2

HPV E6-induced degradation of multi-PDZ protein MUPP1 involves the ubiquitin-proteasome pathway.

The interactions between HPV E6 and the p53 tumour suppressor appear to be central to the antiapoptotic activities of E6 however, as already discussed, the transforming potential of the high-risk HPV E6 protein also relies on its interactions with other cellular targets. In particular a highly conserved C-terminal domain, which is not involved in p53 binding and degradation (Crook et al., 1991b; Pim et al., 1994), has been found to contribute to E6 transforming activity, since its deletion impairs the ability of high-risk HPV E6 to transform rodent cells and immortalise keratinocytes (see introduction). This region contains a PDZ-binding motif.

A general theme that has emerged from studies on DNA tumour viruses is that seemingly unrelated oncoproteins encoded by these organisms often target the same cellular factors. Indeed, the major oncogenic determinant for human Adenovirus type 9, i.e. E4ORF1 owes its oncogenic potential to an intact C-terminal PDZ-binding motif (Weiss et al., 1997; Lee et al., 1997). In the last few years, several PDZ domain-containing proteins have been shown to be common targets for Adenovirus 9 E4ORF1 and high-risk HPV E6: these are the putative tumour suppressor Dlg (Lee et al., 1997; Gardiol et al., 1999), MAGI-1 (Glausinger et al., 2000) and the multi-PDZ protein MUPP1 (Lee S. et al., 2000; this thesis). All these cellular factors are bound by the viral oncoproteins through the last four carboxyl-terminal residues: XT/SXV that comprise a functional PDZ-binding motif. The consequences of these interactions, however, appear to be different in that E4ORF1 simply sequesters its targets into cytoplasmic aggregates thus interfering with their normal functions, while the high-risk HPV E6 proteins induce their degradation through the ubiquitin-proteasome pathway.

HPV-18 E6 binds MUPP1 through its PDZ-binding motif and promotes its degradation in vitro.

We have recently demonstrated that the Adenovirus 9 E4ORF1 (9ORF1) protein binds to MUPP1. MUPP1 has 13 PDZ domains, yet 9ORF1 specifically recognises PDZ domains 7 and 10. As a consequence of this interaction 9ORF1 sequesters MUPP1 in cytoplasmic aggregates (Lee S. et al., 2000). MUPP1 has been shown to localise at tight junctions of polarised epithelial cells (Hamazaki et al., 2002) and its cytoplasmic retention is therefore likely to inactivate its physiological functions.

Because, like 9ORF1, high-risk HPV E6 oncoproteins also possess a functional PDZ-binding motif and complex with Dlg (Lee et al., 1997; Kiyono et al., 1997), we next explored the possibility that they likewise bind to MUPP1. HA-tagged MUPP1 protein was overexpressed in COS7 cells, and subsequently cell lysates were tested in GST-pulldown assays with either GST-9ORF1 or with different GST-E6 fusion proteins. The sequences of wild type and mutant PDZ-binding motifs of 9ORF1 and HPV E6 proteins are shown in table 4.

Protein	X	S/T	X	V/I/L
wt 18 E6	E	T	Q	V
18 E6-V158A	E	T	Q	A
18E6-T156D/V158A	E	D	Q	A
wt 16 E6	E	T	Q	L
wt 11 E6	D	L	L	P
wt 9ORF1	A	T	L	V

Table 4. Alignment of the C-terminal ends of E6 proteins derived from HPV types associated with genital infections and of the Adenovirus 9 E4ORF1 (9ORF1) transforming protein. Shown in red is the consensus binding site for type I PDZ domains (Harris and Lim, 2001). The C-terminal sequences of wild-type high-risk HPV-16 and HPV-18 E6 proteins and the 9ORF1 transforming protein contain a PDZ binding motif, which is not present at the C-terminus of low-risk HPV-11. HPV-18 E6 mutants containing amino acid substitutions are indicated in blue.

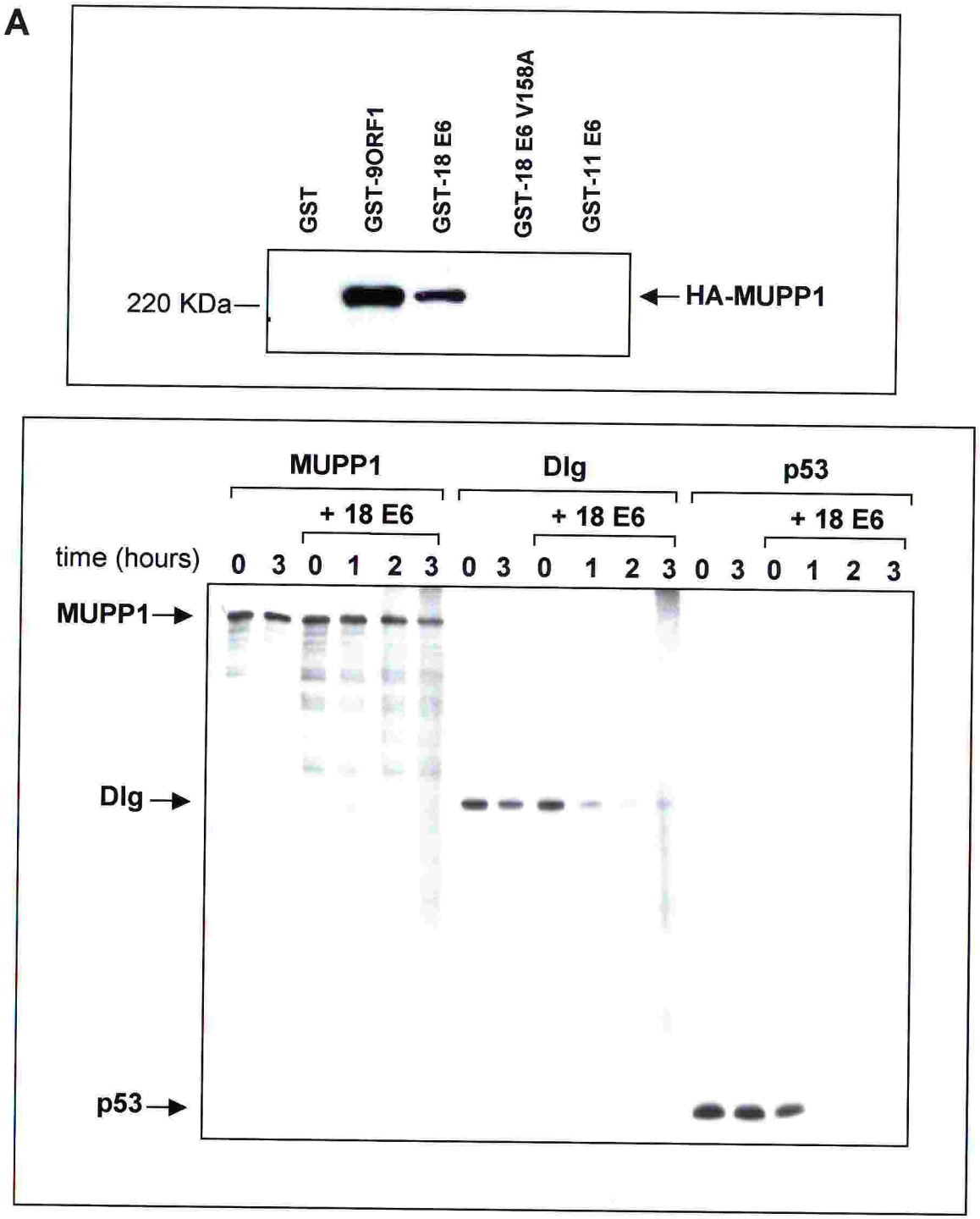


Figure 12. High-risk HPV-18 E6 binds MUPP1 protein and promotes its degradation in vitro.

A. COS 7 cells were lipofected with GW1-HAMUPP1 and cell lysates (200 μg) in RIPA buffer were first subjected to a GST pull down assay with the indicated fusion proteins and then examined by Western blot wit anti-HA antibody. This experiment was performed by S. Lee (Lee S. et al., 2000).

B. *In vitro* translated MUPP1, Dlg or p53 protein was incubated for the indicated times with a five-fold molar excess of *in vitro* translated 18E6 protein or with an equivalent volume of water-primed *in vitro* translation reaction mixture. Proteins from each reaction were subjected to immunoprecipitation with anti-MUPP1, -Dlg and -p53 antibodies, respectively, and detected by autoradiography.

As can be seen from Figure 12A, the wild-type HPV-18 E6 protein associates with HA-MUPP1 protein, and this binding is specific and dependent upon E6 PDZ-binding motif, since a single amino acid substitution (V158A), disrupting the functional PDZ-binding motif of HPV-18 E6, is sufficient to abolish the interaction. Moreover low-risk HPV-11 E6 protein, which lacks a PDZ-binding motif, is also unable to bind MUPP1.
The fact that high-risk HPV E6 oncoproteins promote the degradation of several cellular factors, including the tumour suppressor proteins p53 (Scheffner et al., 1990) and Dlg (Gardiol et al., 1999), prompted us to test whether HPV-18 E6 has similar effects on MUPP1. Incubation of *in vitro* translated high-risk HPV E6 with either p53 or Dlg leads to their degradation (Scheffner et al., 1990; Gardiol et al., 1999), therefore MUPP1 was also examined in similar assays. As shown in Figure 12B, a clear reduction in MUPP1 protein levels was reproducibly observed following a 3-hours incubation with HPV-18 E6, and this effect was consistently greater than that observed in control water-primed *in vitro* translation reactions. However, MUPP1 degradation occurred at a lower extent if compared with that of p53 and Dlg proteins in the same assay.

Expression of HPV-18 E6 reduces both the steady state levels and half-life of MUPP1 protein in vivo.

We next decided to examine if HPV-18 E6 was able to induce MUPP1 degradation also in cells. Therefore we expressed HA-tagged MUPP1 in COS7 cells either alone or together with different E6 proteins, and the cells were lysed 48 hours post transfection and the levels of HA-MUPP1 proteins determined by Western blot. As shown in Figure 13A, cells expressing both MUPP1 and HPV-18 E6 show substantially reduced steady-state levels of MUPP1 protein compared with cells expressing MUPP1 alone. Moreover, this effect was not due to E6 sequestering MUPP1 in insoluble aggregates as observed for 9ORF1 (S. Lee personal communication). The ability of E6 to reduce MUPP1 levels was dependent on the presence of a functional PDZ-binding motif, because HPV-18 E6 mutants V158A and T156D/V158A, as well as low-risk HPV-11 E6

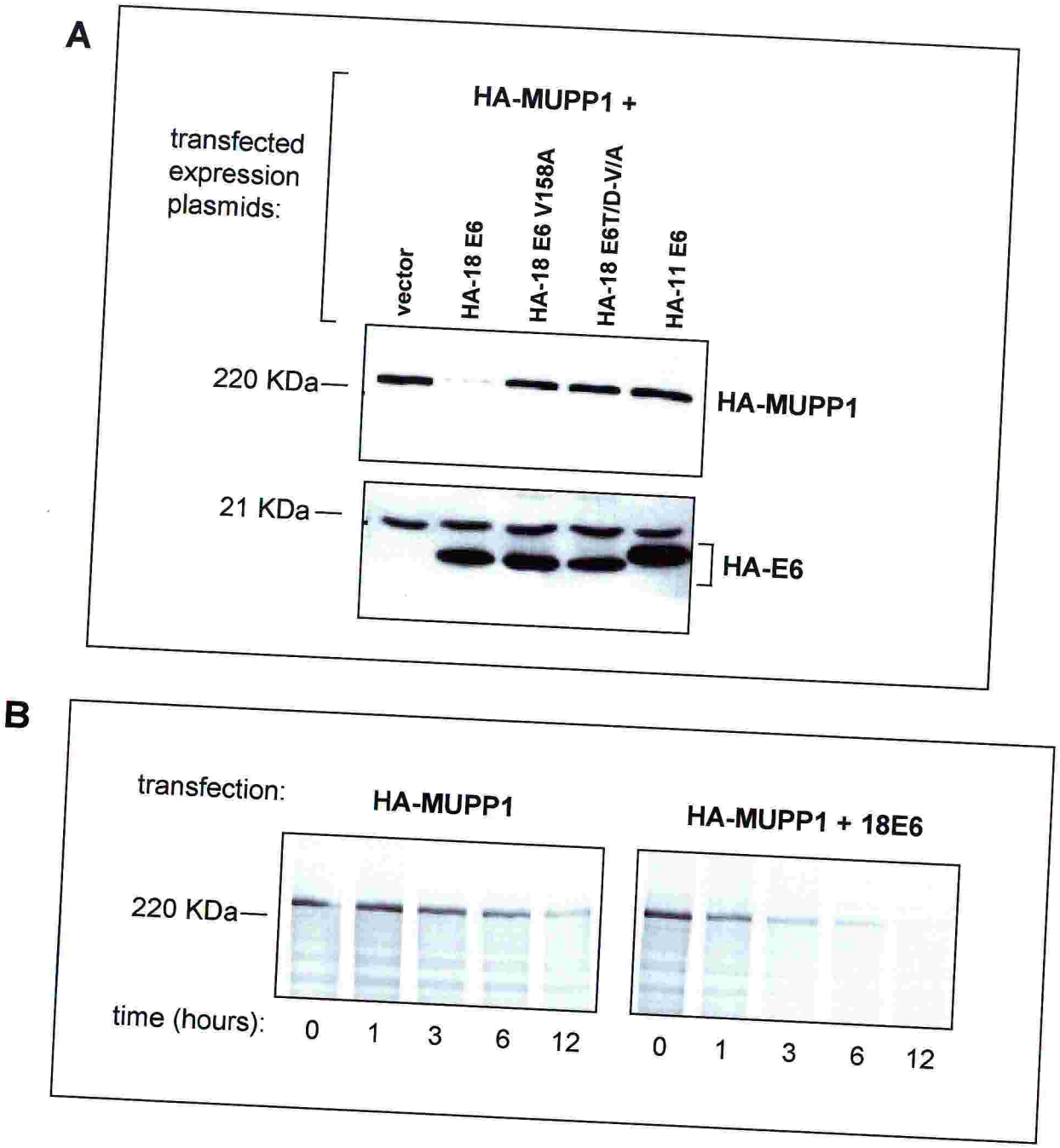


Figure 13. HPV-18 E6 reduces the steady-state levels and the stability of MUPP1 protein in cells.

A) COS 7 cells were lipofected with 1 μg of GW1-HAMUPP1 plasmid and 4 μg of either empty GW1 plasmid (vector) or GW1 plasmid expressing the indicated HA-tagged wild-type or mutant E6 proteins. After 48 hours the cells were lysed and HAMUPP1 and HA E6 proteins detected by Western blot with anti-HA antibodies.

B) COS7 cells were transfected with 1 μg of GW1-HAMUPP1 expression plasmid together with either 4 μg of GW1 empty plasmid or of GW1-HAE6 plasmid. At 24 hours post transfection cells were metabolically labeled for 1 hour with ^{35}S Met/Cys and then chased for the times indicated. Residual HAMUPP1 protein was immunoprecipitated via the HA tag and then assessed by SDS-PAGE and autoradiography. Quantisation of the autoradiograms was performed by PhosphoImager. These experiments were performed by S. Lee (Lee S. et al., 2000).

all lack functional PDZ-binding motifs and concomitantly fail to affect MUPP1 protein levels. To verify that the E6-mediated reduction of MUPP1 protein levels was due to decreased stability of this protein in cells, we performed ^{35}S metabolic labelling and pulse-chase experiments with COS7 cells expressing HA-MUPP1 either alone or together with HPV-18 E6. The residual HA-MUPP1 protein was precipitated with anti-HA antibody at different time points and subsequently examined by SDS-PAGE and autoradiography. The results (Figure 13B) show that MUPP1 protein levels modestly declined after a 3-hours chase period in the absence of E6 whereas they were more reduced after only one hour in the presence of E6. By quantifying the amounts of radioactivity present in MUPP1 protein bands at each time point, we estimated that the half-life of MUPP1 was shortened from 5.7 hours in control COS7 cells to 1.3 hours in HPV-18 E6 expressing COS7 cells. This greater than fourfold decrease in the stability of MUPP1 protein argues that HPV-18 E6 targets this cellular factor for degradation in cells.

Degradation of MUPP1 by HPV-18 E6 in vitro is an ATP-dependent process.

At this point an obvious question is whether E6 targets MUPP1 for ubiquitin-mediated degradation, similarly to other cellular proteins such as p53 and Dlg. The differences in the efficiency of *in vitro* degradation of MUPP1 with respect to the other targets of E6 could imply the usage of a different mechanism, or of a different ubiquitin ligase.

As discussed above, ubiquitin-mediated degradation is an active process that requires the hydrolysis of ATP. Therefore, to test if ATP hydrolysis is required for the E6-induced degradation of MUPP1, a standard *in vitro* degradation assay was performed either in the presence of excess AMP or in the presence of a non-hydrolysable ATP analogue, ATP-γS (Scheffner et al., 1990). As can be seen in Figure 14A, incubation of *in vitro* translated, radiolabelled MUPP1 with HPV-18 E6 in a 1:5 molar ratio, leads to complete destruction of MUPP1 protein within three hours. However, adding either AMP or ATP-γS fully inhibits E6-mediated degradation of MUPP1, thereby proving

that this is an ATP-dependent process. Interestingly, comparing the first two with the last two lanes of Figure 14A, it is evident that ATP-γS can also inhibit the intrinsic, E6-independent degradation of MUPP1. This observation suggests that the reticulocyte lysate contains enzymes that catalyse the ATP-dependent degradation of MUPP1 even in the absence of E6.

MUPP1 is degraded via the proteasome pathway both in the presence and in the absence of high-risk HPV E6.

The data obtained *in vitro* however did not definitively prove the involvement of the ubiquitin-proteasome pathway in degradation of MUPP1 by E6. To directly address this point the HA-tagged MUPP1 expression vector was transfected into 293 cells, both in the presence of a two-fold molar excess of HPV-18 E6 expression vector or of empty vector as a control. After 36 hours, triplicate transfections were either treated for two hours with one of two different proteasome inhibitors, LL and CBZ (see above) or with DMSO as a control. The cells were then lysed and HA-MUPP1 protein levels were analysed by Western blot with anti-HA antibody. As a control, the expression of lacZ control vector was also checked by probing the Western blot with anti-βgalactosidase antibody. As shown in Figure 14B, coexpression of HPV-18 E6 leads to a massive reduction in the steady-state levels of MUPP1 protein, which is almost undetectable in untreated cells. However, inhibition of the proteasome activity completely rescues the levels of MUPP1 protein up to those of the control-transfected cells, clearly demonstrating that the proteasome pathway is fully responsible for mediating the destruction of MUPP1 induced by HPV-18 E6. Interestingly, this experiment also confirmed the results obtained from the *in vitro* degradation assay, since proteasome inhibition led to an increase in the amount of MUPP1 protein even in the absence of E6, and this would argue that proteasome-mediated degradation of MUPP1 can occur through both E6-dependent and –independent pathways.

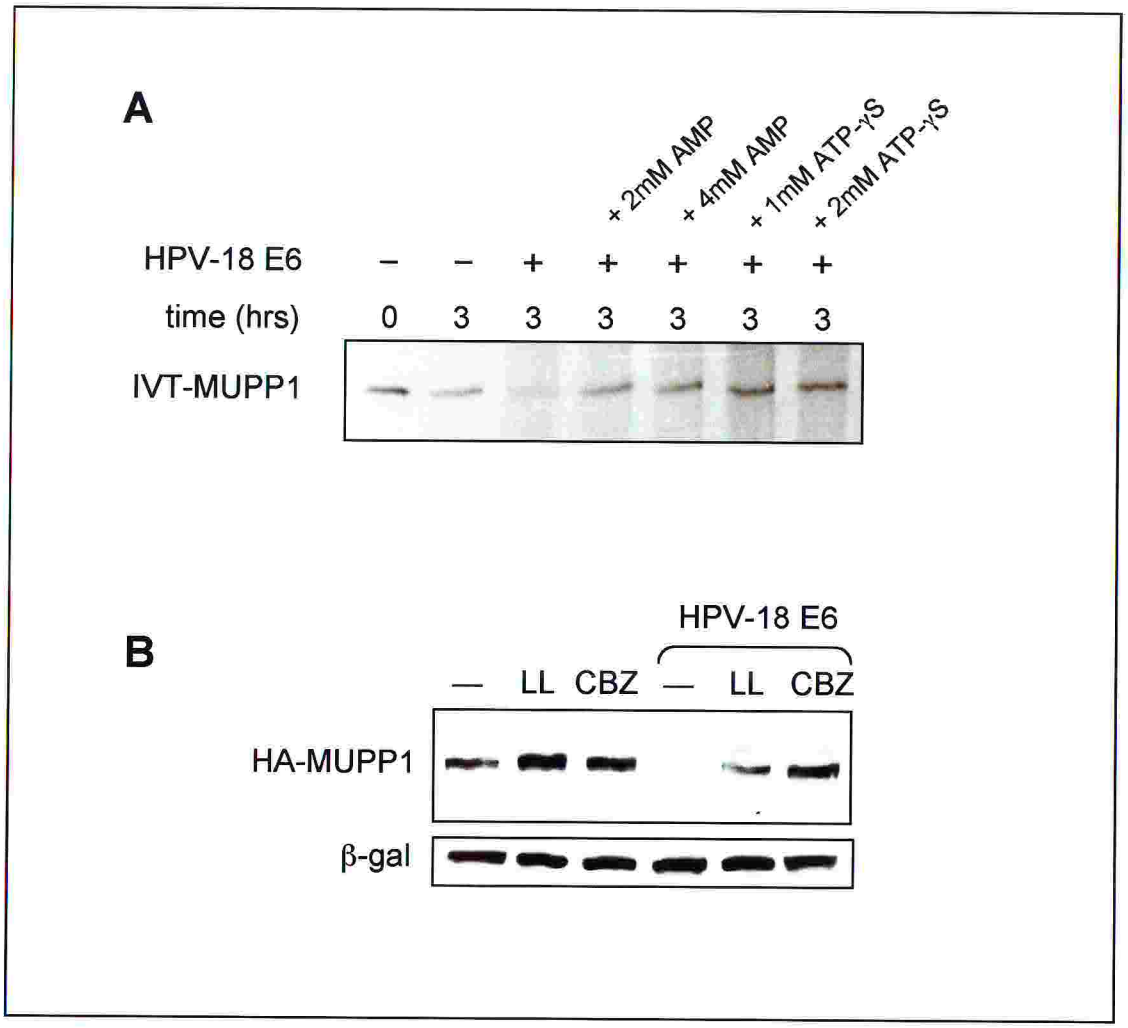


Figure 14. High-risk HPV-18 E6 causes degradation of MUPP1 protein by the proteasome pathway.

(A) HPV-18 E6 promotes the ATP-dependent proteolysis of MUPP1 protein *in vitro*. *In vitro* translated (IVT), radiolabelled MUPP1 was incubated for the indicated times with a fivefold molar excess of *in vitro* translated HPV-18 E6 protein (+) or with an equivalent volume of a water-primed *in vitro* translation reaction mixture (-). An excess of AMP or of the non-hydrolysable ATP analog, ATP-γS was added to the mixture where indicated. After three hours of incubation, MUPP1 protein was immunoprecipitated with MUPP1 specific antiserum and detected by autoradiography.

(B) Proteasome inhibition counteracts the reduction of MUPP1 steady-state levels *in vivo*. 293 cells were transfected with 2 μg of HA-MUPP1 expression vector together with 4 μg of either HPV-18 E6 expression plasmid or of empty vector as a control. After 36 hours the cells were either treated with the proteasome inhibitors N-acetyl-leu-leu-Norleucinal (LL) or N-CBZ-leu-leu-leu-al (CBZ) or with DMSO as a control for two hours and then harvested. HA-MUPP1 protein was detected by anti-HA Western blot. Probing of the Western blot for β-galactosidase expression is shown in the panel below and confirms equal levels of transfection efficiency.

Sequences in the carboxyl-terminal half of HPV-18 E6 are required for the degradation of MUPP1.

Previous studies had defined the regions of HPV E6 required for binding and degradation of p53 (Crook et al., 1991; Pim et al., 1994; Li and Coffino, 1996), and of Dlg (Pim et al., 2000). We have previously found that the PDZ-binding motif of high-risk HPV E6 is absolutely required for binding and consequently for degrading MUPP1. However, other domains of the protein are also likely to be involved, for instance in binding to the ubiquitin ligase and/or to other cellular factors responsible for ubiquitination. In order to determine if the regions of E6 that are involved in MUPP1 degradation are overlapping with those responsible for the destruction of either p53 or Dlg, a panel of previously characterised HPV-18 E6 mutant proteins was used, which contain a series of short deletions (Pim et al., 1994; Pim et al., 2000): these are shown schematically in Figure 15B. A degradation assay was then performed *in vivo* as described above, but this time MUPP1 and each E6 expression plasmid were cotransfected both in a 1:1 and 1:2 molar ratio, to better allow discrimination of quantitative variations in the effects of different E6 proteins. As can be seen from Figure 15A, a domain of E6 that appears to be absolutely required for degradation of MUPP1 encompasses a C-terminal portion of the HPV-18 E6 protein spanning residues 101 to 117, as defined by mutants ΔE (Δ101-104) and ΔF (Δ113-117) in the C-terminal loop. These two mutant E6 proteins appear to have completely lost the ability to target MUPP1 for degradation, even though the deletions lie far from the MUPP1 binding motif, which is located at the extreme C-terminus of the E6 protein (residues 155-158). Interestingly mutant ΔH (Δ144-149), which lacks six amino acid residues immediately upstream of the PDZ-binding motif, thereby bringing this motif closer to the C-terminal loop, appears to degrade MUPP1 with an efficiency even higher than that obtained with the wild-type 18 E6 protein. It is possible that this deletion, which reduces the spacing between the zinc finger and the MUPP1-binding domain, alters E6 protein conformation thus increasing its affinity for either MUPP1 or the ubiquitin ligase. The ΔD mutation (Δ21-25) upstream of the first zinc finger

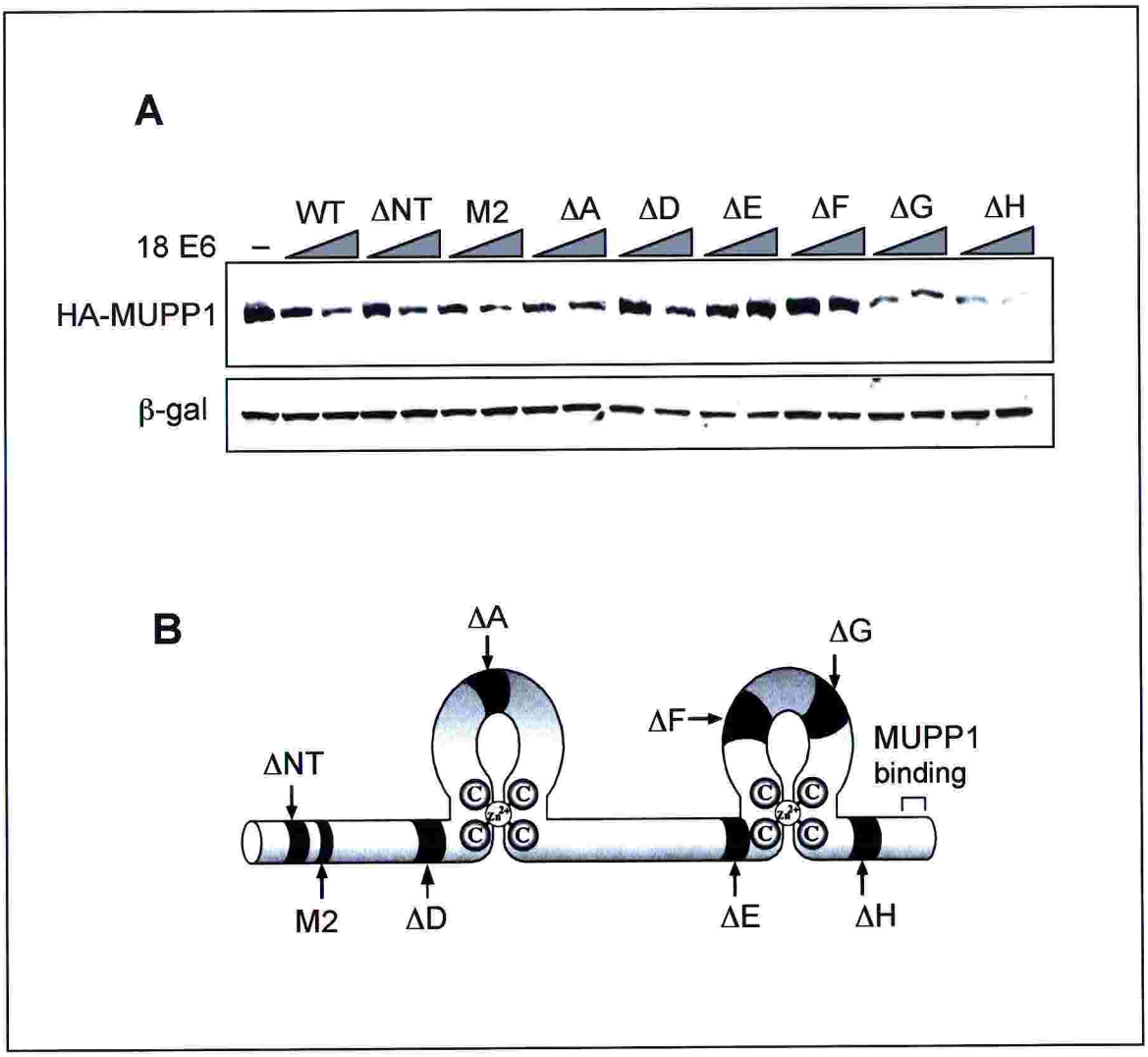


Figure 15. Mutational analysis of the regions of HPV-18 E6 involved in inducing the degradation of MUPP1 *in vivo*.

(A) 293 cells were transfected with 2 μg of the HA-MUPP1 expression vector and either 2 or 4 μg of the wild type (wt) and mutant HPV-18 E6 expression plasmids. After 36 hours the cells were harvested and the residual MUPP1 protein ascertained by anti-HA Western blot analysis. Probing of the Western blot for β-galactosidase expression is shown in the panel below and confirms equal levels of transfection efficiency.
(B) Schematic diagram showing the location of the mutations in HPV-18 E6 used in this study: ΔNT (Δ4–7), M2 (R10S, P11G), ΔD(Δ21–25), ΔA(Δ47–49), ΔE (Δ101–104), ΔF (Δ113–117), ΔG (Δ126–130), ΔH (Δ144–149). For comparison the position of the core MUPP1 binding domain is also shown (residues 155-158).

appears to partially impair MUPP1 degradation, while ΔNT (Δ4-7) deletion has no effect. The results obtained with the other amino-terminal mutants are also interesting, since mutants M2 (R10S, P11G) and ΔA (Δ47-49) have been shown to be defective with respect to their ability of inducing p53 degradation (Pim et al., 1994; Gardiol and Banks, 1998), however they are both active with respect to Dlg degradation (Pim et al., 2000), and as can be seen from Figure 15A, they also appear to be active with respect to the degradation of MUPP1. Therefore it can be concluded that the regions of E6 which are involved in degradation of p53 are not required for degradation of MUPP1, and this would imply that the mechanisms used by E6 in these two cases are different.

The presence of a functional PDZ-binding motif mediates MUPP1 degradation by different E6 molecules.

To better define the mechanism by which the HPV E6 proteins target MUPP1 for degradation, different wild-type E6 proteins plus a previously characterised panel of chimaeric E6 molecules were used (Pim et al., 2000). Previous studies had shown that both the amino- and carboxyl-terminal halves of the high-risk HPV E6 proteins are required for the degradation of p53 (Crook et al., 1991b; Pim et al., 1994; Li and Coffino, 1996). In particular, the use of HPV-6 and HPV-16 E6 chimaeric molecules showed that sequences in the carboxyl-terminal half of E6 are largely responsible for binding p53, whereas sequences in the amino-terminal half of E6 are responsible for degradation (Crook et al., 1991b; Li and Coffino, 1996). Therefore, HA-tagged MUPP1 was cotransfected with the different E6 expression vectors in either 1:1 or 1:2 molar ratio, and subsequently the steady-state levels of MUPP1 were analysed. The results of this experiment are shown in Figure 16A. Comparing the efficiency by which the different wild-type E6 proteins target MUPP1 for degradation, it can be observed that the low-risk HPV-11 E6 protein, which lacks the PDZ-binding motif, is not able to induce MUPP1 degradation confirming previous results. In addition, HPV-18 E6

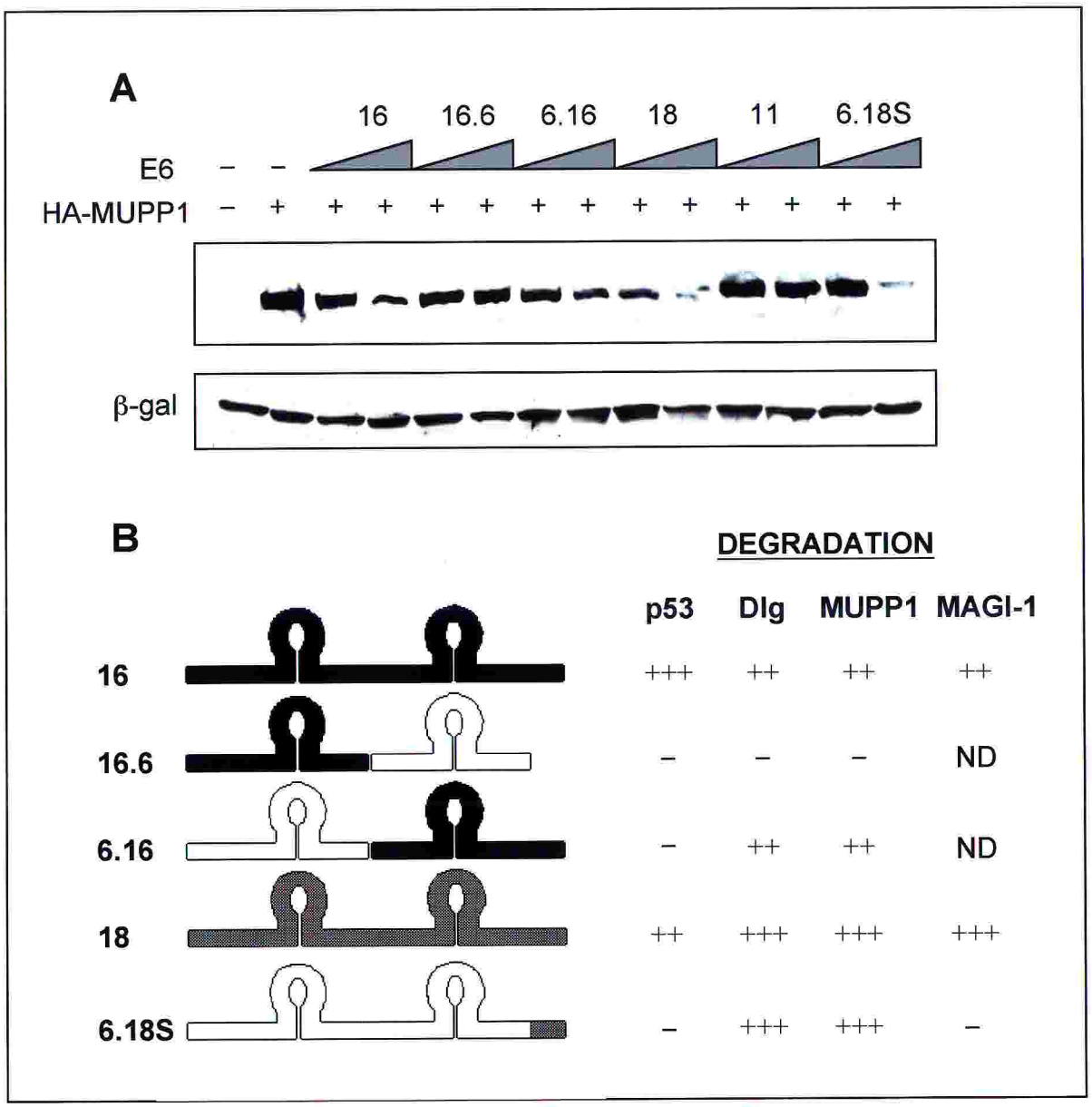


Figure 16. Addition of the PDZ-binding motif of HPV-18 E6 onto HPV-6 E6 confers the ability to target MUPP1 protein for degradation.

(A) 293 cells were transfected with 2 μg of HA-MUPP1 expression plasmid together with 2 μg of either the wild type or chimaeric E6 expression plasmids as indicated. After 36 hours the cells were harvested and residual MUPP1 protein was ascertained by anti-HA Western blot. Probing of the Western blot for β-galactosidase expression is shown in the panel below and confirms equal levels of transfection efficiency.
(B) Schematic diagram showing the nature of the chimaeric HPV E6 proteins and their activity with respect to degradation of p53, Dlg, MAGI-1 and MUPP1. Black portions represent HPV-16 E6 derived sequences, white portions represent HPV-6 E6 derived sequences and gray portions represent HPV-18 E6 derived sequences. The 6.16 chimaera comprises the N-terminal 60 residues from low-risk HPV-6 E6 and the C-terminal 92 residues from HPV-16 E6. The 16.6 chimaera contains the N-terminal 59 residues from HPV-16 E6 and the C-terminal 90 residues from HPV-6 E6. (Crook et al., 1991b). The 6.18S chimaera contains the entire HPV-6 E6 sequence with the last 7 residues of HPV-18 E6 fused to its C-terminus (Pim et al., 2000).

appears to be more efficient than HPV-16 E6 in degrading MUPP1, similarly to what has been reported for Dlg (Pim et al., 2000) and MAGI-1 (Thomas et al., 2001). Particularly interesting however, are the results obtained with the chimaeric E6 molecules, whose structure is schematically represented in Figure 16B. As can be seen from figure 16A the HPV 6.16 protein is as effective as the full-length HPV-16 E6 in targeting MUPP1 for degradation, while the 16.6 chimaera has no effect on MUPP1 steady-state levels. This is consistent with the above results showing that all the sequences required for MUPP1 degradation are contained in the second half of the high-risk E6 molecule. Most interestingly, the addition of only the last seven amino acids of high-risk HPV-18 E6 onto the carboxyl-terminus of low-risk HPV-6 E6 confers the ability to degrade MUPP1 with an efficiency comparable to that of full-length HPV-18 E6. This result implies that, if provided with a functional PDZ-binding motif, low-risk HPV-6 E6 protein otherwise contains all the necessary sequences required for mediating the degradation of MUPP1.

DISCUSSION

It has been reported that expression of high-risk HPV E6 in mammalian cells is able to disrupt the integrity of epithelial tight junctions, and a functional PDZ-binding motif was shown to be essential for this activity of E6 (Nakagawa and Huibregtse, 2000). This strongly suggests that combined inactivation of different tight-junction PDZ proteins such as MUPP1, MAGI-1 and hScrib by E6 might contribute towards the loss of epithelial cell adhesion and polarity of HPV-associated cancers. MUPP1 has the capacity to assemble a large array of cellular proteins into a multitude of different signalling complexes beneath the plasma membrane at tight junctions. These protein clusters might play important roles in signal transduction pathways regulating the growth and/or differentiation of epithelial cells. Indeed, the fact that two unrelated viral transforming proteins such as Ad9 E4ORF1 and high-risk HPV E6 have both evolved to target the MUPP1 protein, is in favour of a putative role for MUPP1 in regulating crucial aspects of cell biology such as proliferation or maintenance of cell polarity. In this part of the study the E6 proteins of high-risk HPV-16 and HPV-18 have been shown to target MUPP1 for proteasome-mediated degradation *in vivo*. In contrast the low-risk HPV-11 E6 protein, which lacks the PDZ-binding motif, is completely unable to bind MUPP1 and to induce its degradation. These results are analogous to what has been shown for other PDZ-containing proteins such as Dlg (Lee et al., 1997; Gardiol et al., 1999), MAGI-1 (Glausinger et al., 2000) and hScrib (Nakagawa et al., 2000). Moreover, we observed that HPV-18 E6 is apparently more efficient than HPV-16 E6 in degrading MUPP1, and this is most likely due to the different sequences of their PDZ-binding motifs: these are ETQV for HPV-18 E6 and ETQL for HPV-16 E6 (see Table 4). Indeed, these results are in agreement with previous reports showing that HPV-18 E6 also binds and degrades both Dlg and MAGI-1 more efficiently than HPV-16 E6, and that the single amino acid difference between their PDZ-binding motifs is in fact responsible for this behaviour (Pim et al., 2000; Thomas et al., 2001). This is in marked contrast with the E6-p53 interaction, where HPV-16 E6 has consistently been shown to bind and degrade p53 more

effectively than HPV-18 E6 (Werness et al., 1990; Scheffner et al., 1990) A summary of the activities of the different E6 proteins on their targets p53, Dlg, MUPP1 and MAGI-1 is shown in Figure 16B. It is interesting to speculate whether the higher activity of HPV-18 E6 with respect to the PDZ-containing proteins may be reflected in any pathological outcomes of infection. Certainly, there is an extensive literature showing that HPV-18 is generally more active than HPV-16 in a variety of transformation assays (Barbosa and Schlegel, 1989; Villa and Schlegel, 1991). In addition, there are also several reports which indicate that HPV-18 containing cervical tumours have a more aggressive phenotype and are also more prone to recurrence than those containing HPV-16 (Burnett et al., 1992; Kurman et al., 1988; Zhang et al., 1995). The only known biochemical activity of HPV-18 E6 which correlates with this trend, so far, is its high affinity for cellular targets containing PDZ domains, and all these findings have important implications for the role of the interaction of high-risk HPV with MUPP1 in the development of cervical cancer. In addition, comparison of the effects of HPV-18 E6 protein on different cellular substrates such as MUPP1, Dlg and p53 highlights a hierarchy of susceptibility to E6-mediated degradation among these proteins. These observations could imply the usage of different pathways by high-risk HPV E6 to target different cellular substrates. It is notable that, while the rate of MUPP1 degradation *in vitro* is quite low compared with other substrates of E6, nonetheless it appears to be efficiently degraded *in vivo*. Marked differences in the susceptibility of p53 mutant proteins to E6-mediated degradation between *in vitro* and *in vivo* assays have been previously described (Crook et al., 1996; Gardiol and Banks, 1998) and there are several possible explanations for this. It is possible that a multisubunit ubiquitin ligase is involved in MUPP-1 degradation and that some components are limiting *in vitro*. Alternatively, binding to the substrate protein *in vitro* may be inefficient, either due to incorrect folding of the *in vitro* translated proteins, or the multi-PDZ domain protein may form oligomers. In addition, since many proteins are rendered susceptible to ubiquitination through phosphorylation, we cannot exclude the possibility that posttranslational modifications may be required for MUPP1 to be degraded. To further investigate the mechanism by which

E6 induces the degradation of MUPP1, a number of mutant E6 proteins were then analysed. The results demonstrated that the N-terminal sequences of high-risk HPV E6 that are needed for degradation of p53 (Li and Coffino, 1996) are not required for degradation of MUPP1. From our experiments it appears that only a short sequence in the carboxyl-terminal loop of HPV-18 E6 (defined by mutants ΔE and ΔF) is, together with the PDZ-binding domain, absolutely required for MUPP1 degradation. Interestingly, an overlapping region of HPV-18 E6 spanning residues 101-130 has previously been found to be required for degradation of Dlg (Pim et al., 2000). Taken together, these data indicate that very similar regions of the high-risk HPV-18 E6 protein are required to target MUPP1 and Dlg for degradation, and this would lead to the hypothesis that these two processes follow the same pathway. Moreover, our data also suggest that this pathway is separate from that used for degrading p53, and raise the possibility that different ubiquitin ligases are also involved. Indeed it has been shown that the low-risk HPV E6 proteins have very low affinity for E6AP (Huibregtse et al., 1991; Huibregtse et al., 1993b; Nakagawa and Huibregtse, 2000) and, not surprisingly, the HPV-6.16 chimaeric E6 protein, containing the N-terminal half of low-risk HPV E6 protein fused to the C-terminal part of high-risk HPV E6 molecule, is unable to degrade p53 (Pim et al., 2000). However, the 6.16 chimaeric E6 protein is able to induce MUPP1 degradation with the same efficiency as the wild-type high-risk HPV-16 E6 protein and this observation would apparently rule out a role for E6AP in this process. This is consistent with the observation that Dlg also appears to be targeted for proteasome-mediated degradation by E6 through an E6AP-independent pathway, since it is also degraded by the HPV 6.16 E6 chimaera (Pim et al., 2000). Interestingly, MAGI-1 is not degraded by this chimaeric protein (D. Pim personal communication), suggesting that the protein complexes governing the degradation of this substrate are likely to be different from those required for Dlg. A schematic diagram showing degradation of different E6 target proteins by HPV E6 chimaeric proteins is shown in Figure 16B. As well as showing that the N-terminal part of a low-risk HPV E6 protein is capable of directing MUPP1 degradation when fused to high-risk HPV E6 C-terminal sequences, these studies also demonstrated that a low-risk

HPV E6 protein, when provided with a PDZ-binding motif, is also able to induce degradation of MUPP1 with the same efficiency as full-length high-risk HPV E6 proteins. This result implies that the low-risk HPV E6 proteins also contain sequences that mediate interaction with a cellular ubiquitin ligase. This finding has several interesting implications. Indeed, we have already discussed that the structural characteristic that most clearly distinguishes the high-risk from the low-risk HPV E6 proteins is the presence of the C-terminal PDZ-binding motif, which is essential for their transforming activity, and it could be speculated that such a domain had been acquired by the high-risk viruses from the cellular genome during the course of evolution. Indeed, a similar C-terminal consensus motif is a common feature of many cellular proteins that interact with PDZ-proteins, and intriguingly the PDZ-binding domain of the HPV-18 E6 protein contains a functional PKA phosphorylation site that mediates its regulation by a cellular signalling pathway (Kühne et al., 2000). This finding, together with the position of the PDZ-binding domain at the extreme C-terminus of the E6 molecule renders plausible this hypothesis. Interestingly our observations, together with those previously reported (Pim et al., 2000), indicate that the high- and low-risk HPV E6 proteins share the ability to interact with components of the ubiquitin pathway and therefore to target other proteins for proteasome-mediated degradation. A search for the cellular targets of the low-risk HPV E6 proteins as well as their ubiquitin ligase partners would be of great interest in further understanding the biology of these viruses, which is still poorly defined compared with that of their highly oncogenic counterparts. Finally, it is interesting to note that MUPP1 is also intrinsically regulated by the ubiquitin-proteasome pathway in the absence of HPV E6. The fact that its abundance needs to be regulated by such a specific and highly efficient system would suggest that the role of MUPP1 in epithelial cells is not merely structural, rather it strengthens the hypothesis of an active role of MUPP1 in signalling, as indicated by recent studies (Mancini et al., 2000; Kimber et al., 2002). A better understanding of MUPP1 functions and of their regulation in the cell would be of great interest, and it would also help to clarify the significance of its inactivation for the life cycles of different transforming viruses.

PART 3

Proteasome mediated regulation of the hDlg tumour suppressor protein in epithelial cells.

In the previous section studies on E6 interaction with the PDZ-containing MUPP1 protein were described. However, the best characterised PDZ domain-containing target of E6 to date is Dlg. As already discussed, Dlg is a tumour suppressor protein intimately involved in the control of cell contact, cell polarity and proliferation. It is now well established that the high-risk HPV E6 proteins can promote its degradation through the proteasome pathway, and that this activity most likely contributes to HPV-induced malignancy. However at present, no information is available concerning the levels of hDlg expression in cervical cancer cells containing endogenous HPV E6 protein, and moreover very little is known about the regulation of the endogenous hDlg protein in normal and transformed epithelial cells. Interestingly, previous experiments had indicated that when overexpressed, Dlg is ubiquitinated and degraded by the proteasome pathway even in the absence of HPV E6 (Gardiol et al., 1999; Kühne et al., 2000). These data prompted us to investigate the role of the ubiquitin-proteasome pathway in the regulation of endogenous hDlg protein in a variety of epithelial cell lines, either containing high-risk HPV sequences or not.

hDlg is degraded by the proteasome in epithelial cancer cells.

In order to investigate the effects of E6 upon hDlg in cells derived from HPV-positive cervical tumours, the steady state levels of hDlg protein were compared in the absence and presence of proteasome inhibitors. This was done first on the HPV-16 positive CaSKi cell line. After two hours of incubation with the different proteasome inhibitors N-acetyl-leu-leu-norleucinal (LL), N-CBZ-leu-leu-leu-al (CBZ) and lactacystin (LC), equal amounts of whole cell extracts (50 µg) were separated by SDS-PAGE and analysed by Western blot. hDlg protein was detected with a polyclonal

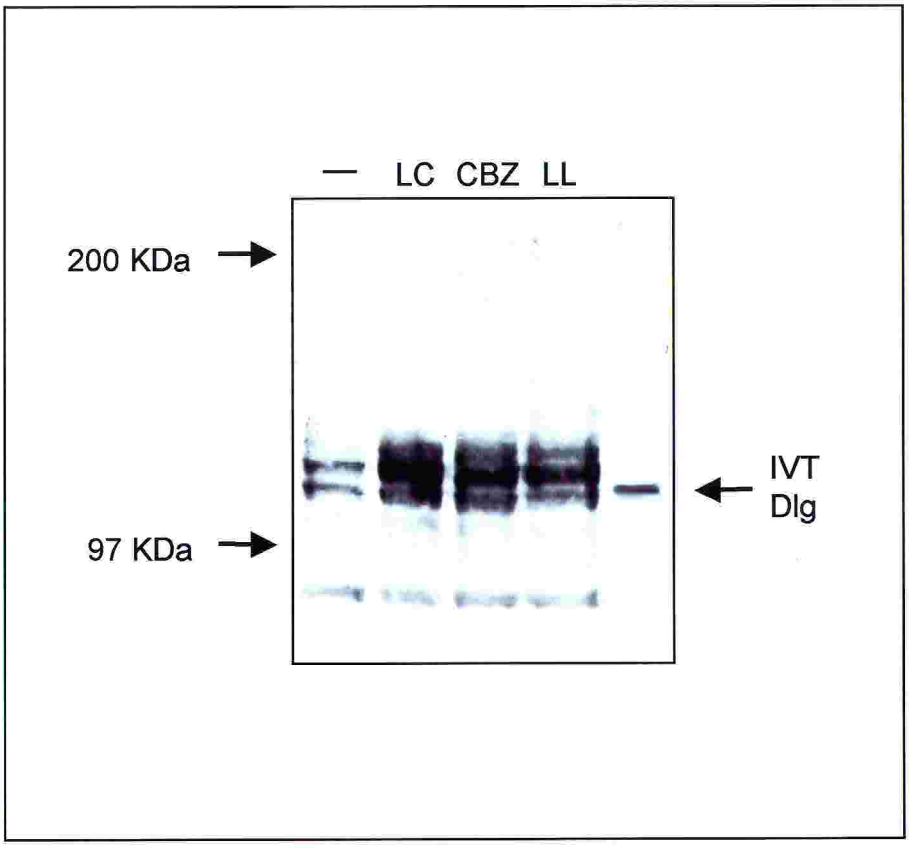


Figure 17. Regulation of hDlg protein by the proteasome pathway in HPV-16 positive CaSKi cervical cancer cells.

CaSKi cells were treated with the proteasome inhibitors lactacystin (LC), N-CBZ-leu-leu-leu-al (CBZ), and N-acetyl-leu-leu-norleucinal (LL), or with DMSO as a control (-) for two hours before harvesting. Equal amounts of protein extracts were then separated by PAGE and analysed by Western blotting with anti-Dlg antiserum. *In vitro* translated Dlg (IVT Dlg) was included as a positive control and the migration of standard molecular weight protein markers is shown. Nonspecific background staining confirms equal loading.

antibody raised against the Dlg N-terminus, which comprises Dlg specific sequences (residues 1-222) and shares no homology with other family members. As can be seen from Figure 17, in control-treated cells the antiserum recognises two major bands, which comigrate with *in vitro* translated Dlg protein. Upon treatment with all three proteasome inhibitors, both forms of hDlg are clearly stabilised, indicating that the endogenous hDlg protein is degraded through the proteasome pathway in HPV-16 positive CaSKi cells. Moreover, additional slower-migrating bands also become evident upon proteasome inhibition, suggesting that these forms of hDlg are more susceptible to degradation.

It has previously been shown that HPV-18 E6 is more efficient in hDlg degradation than HPV-16 E6 (Gardiol et al., 1999; Pim et al., 2000), therefore it was of interest to compare both the levels of hDlg and its regulation by the proteasome in HPV-16 positive CaSKi cells and in HPV-18 positive HeLa cells. The cells were incubated with CBZ at a concentration of 50 μM for either two or four hours, and hDlg protein levels were then monitored as above. The results are shown in Figure 18A. 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 with 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 the proteasome inhibitors to be 100% effective, this lower accumulation would suggest a lower expression level coupled 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 binds and degrades hDlg more efficiently than HPV-16 E6. However, incomplete stabilisation of hDlg could also imply a breakdown of its regulation in HeLa cells by another, as yet unknown, mechanism. Interestingly, the higher molecular weight forms of hDlg that become stabilised in CaSKi cells upon proteasome inhibition are not detected in HeLa cells.

This analysis of hDlg levels and its regulation was then extended to other cervical cancer cell lines containing other high-risk HPV types. Figure 18B shows hDlg

Western blots of several HPV-positive cervical cancer cells, treated with either CBZ proteasome inhibitor for two hours or with DMSO as a control. As can be seen, the amount of hDlg is relatively high in HPV-16 containing CaSKi and SiHa cells even without CBZ treatment, and it is consistently stabilised following proteasome inhibition. hDlg levels are generally lower in cells containing HPV-18 (C4-I, SW756 and HeLa), consistent with the reported higher efficiency of HPV-18 E6 induced hDlg degradation. There is, however, considerable variability in the basal levels of hDlg as well as in the extent of stabilisation achieved upon proteasome inhibition in these HPV-18 containing cells. The highest levels are present within C4-I cells while much less hDlg can be seen in SW756 and HeLa cells. Such variation could reflect differences in the hDlg gene expression levels as well as variability in the rate of hDlg degradation among the different cell lines.

Having demonstrated that the 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 non-tumourigenic immortalised skin keratinocytes and C33I cervical carcinoma cells. Figure 18A shows that HaCaT cells contain relatively high amounts of hDlg, however the protein also becomes efficiently stabilised upon proteasome inhibition. Since there is no HPV E6 protein in these cells, proteasome mediated degradation of hDlg must be regulated here solely by cellular factors. In contrast in C33I cells, which harbour much lower levels of hDlg, very little stabilisation is obtained following proteasome inhibition. Therefore it appears that hDlg is normally subjected to proteasome degradation in epithelial cells, however both the basal protein levels and the extent of degradation are variable between different cell lines.

Since this could be in part a consequence of different rates of transcription, the hDlg mRNA expression levels were then analysed in the different cell lines. Total RNA was extracted from CaSKi, HeLa, HaCaT and C33I cells and reverse transcription followed by a small number of PCR cycles were performed, using specific hDlg primers; Southern blot with an internal probe was then used to detect the products. As a control, TBP (TATA-binding protein) mRNA was also reverse-transcribed and

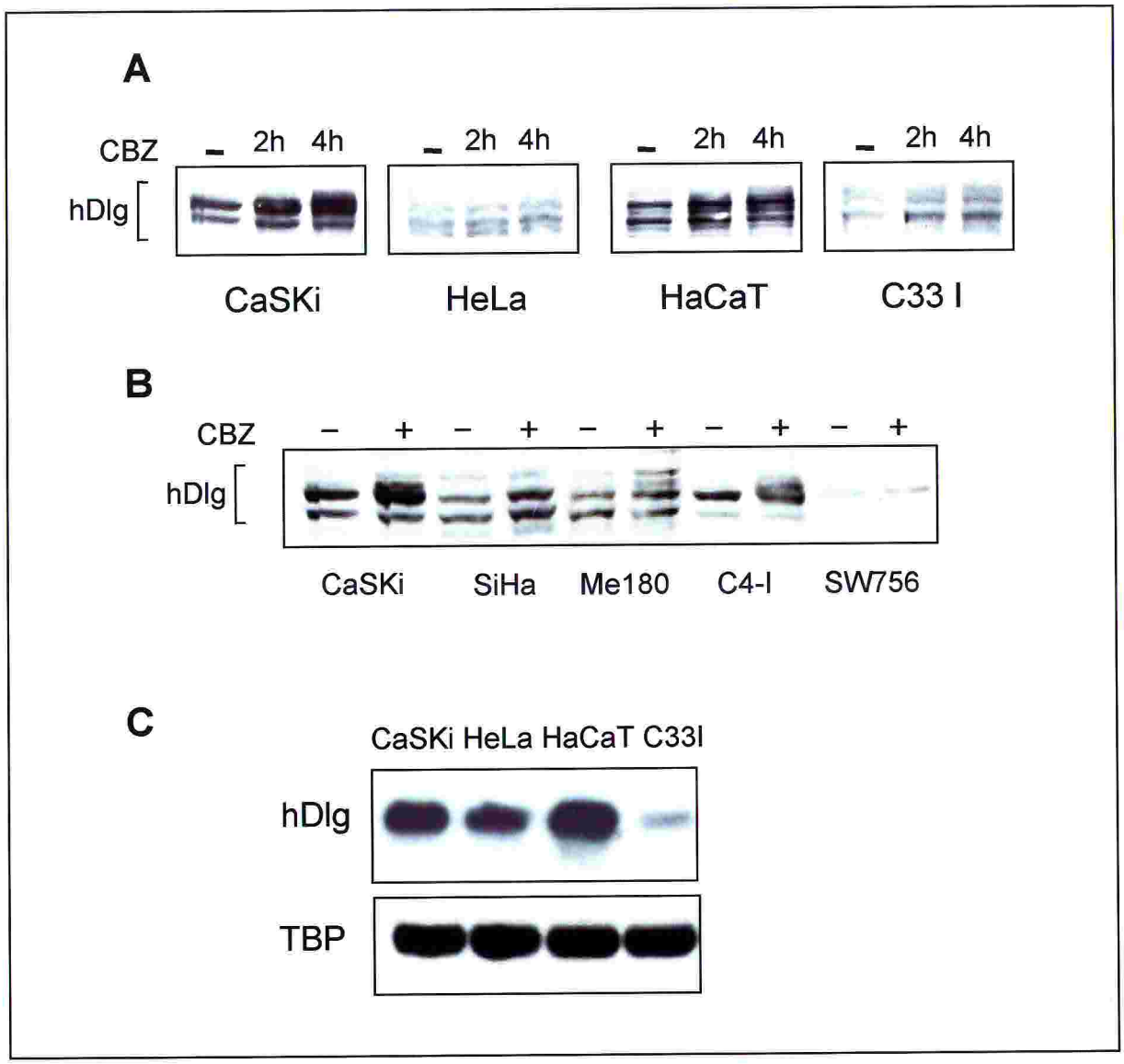


Figure 18. Degradation of hDlg protein by the proteasome pathway in epithelial cancer cells.

(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.
(B) HPV-16 positive CaSKi and SiHa, HPV-68 positive Me180, and HPV-18 positive C4-I and SW756 cells were treated with CBZ for two hours and analysed as above.
(C) 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.

amplified from the same samples. Figure 18C shows that the hDlg mRNA levels do indeed vary among different cell lines. In particular, HaCaT cells show the highest mRNA levels while C33I appear to have the weakest expression, and this correlates with the basal levels of hDlg protein expression. It is also clear from this analysis that in those cells which contain very low levels of hDlg (due 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 expression, suggesting that hDlg protein degradation proceeds at a higher rate in HeLa cells.

hDlg protein is stabilised as a consequence of increased cell contact.

The meaning for these differences in the regulation of hDlg among the various cell lines was intriguing, and analysis of the cellular morphology provided a first indication. As can be seen from Figure 19, those cells which harbour higher levels of hDlg that increase consistently following proteasome inhibition, such as CaSKi and HaCaT cells, retain an epithelial morphology and grow in structured sheets, held together by tight contacts between the cells. In contrast, those cell lines which contain very little hDlg and are less responsive to proteasome inhibition, such as HeLa and C33I cells, show the most transformed phenotypes: these are round or fibroblast-shaped cells, which grow disorderly and form very loose, if any, cell-cell contacts. This is reminiscent of the phenotypes described for *Dlg* 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, their ability to grow in soft agar was tested and the results are shown in Table 5. Interestingly, when cultured in DMEM supplemented with 0.5% noble agar, only HeLa and C33I cells were able to form colonies, while CaSKi and HaCaT, which retain remarkably higher hDlg levels, were unable to proliferate.

It has been reported that 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 very 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 the extent of hDlg degradation was monitored 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 proteasome inhibitor or DMSO as a control. The cell cycle profiles were also analysed by FACS on both the DMSO- and the CBZ-treated cells, in order to confirm that the cells were still growing normally and that no cell cycle arrest had begun to occur at even the highest cell density (data not shown). The results of the Western blots are shown in Figure 19. In both CaSKi and HaCaT cells, it is evident that hDlg abundance is lowest at minimum cell density, while it increases as the cells become more confluent. The stabilisation obtained upon treatment with the proteasome inhibitor CBZ 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 very few contacts with each other. Indeed, at this stage proteasome inhibition completely rescues 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 progress towards confluence and establish an increasing number of cell contacts. Noteworthy, this density-dependent stabilisation of hDlg is far more efficient in CaSKi and HaCaT cells than in HeLa and C33I cells, which have a highly transformed morphology and, even at high cell density, make very few cell contacts. In both CaSKi and HaCaT cells previously undetectable slower migrating forms of hDlg protein become visible at high cell density, suggesting that these forms might have a specific function in the

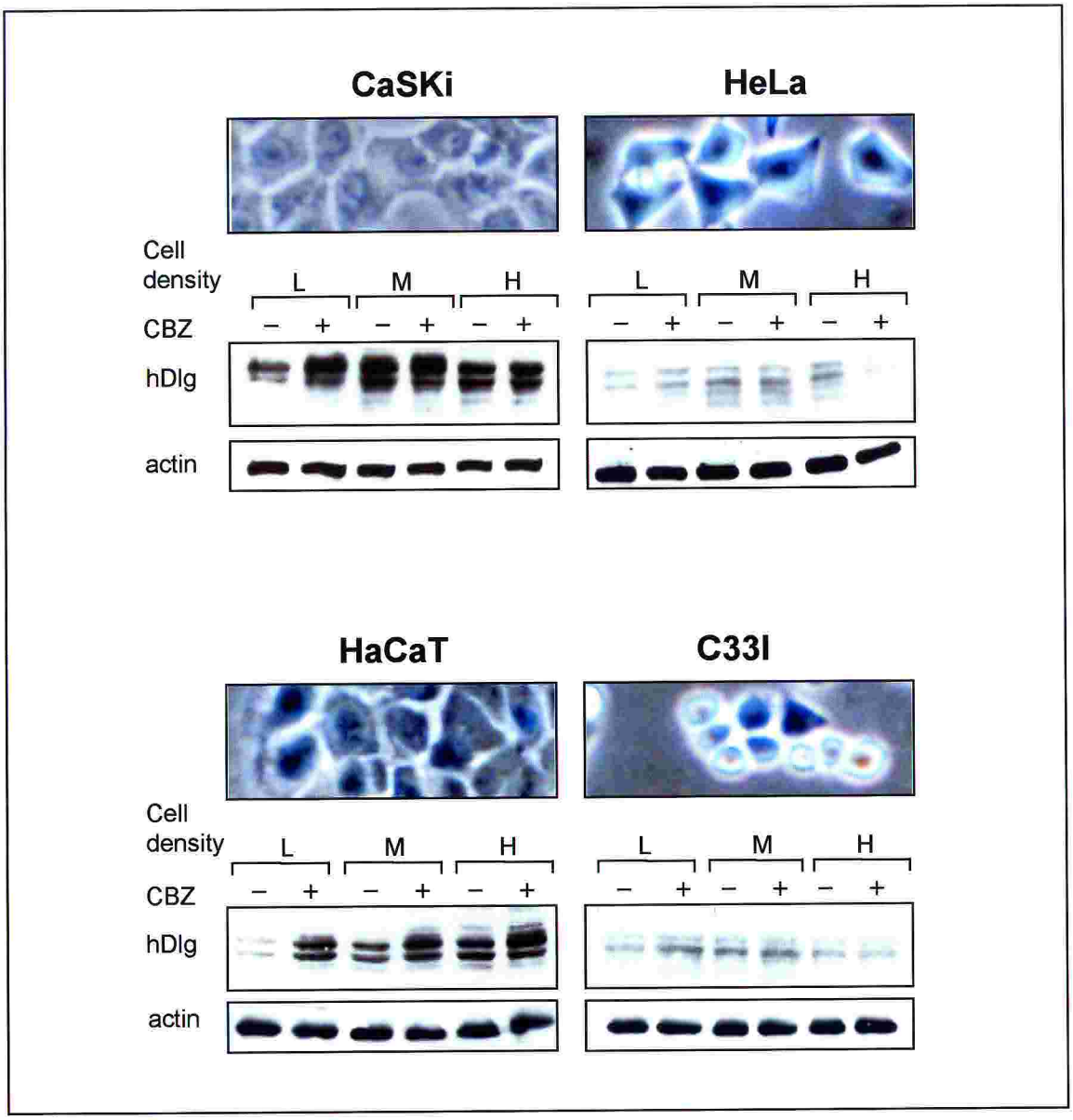


Figure 19. Density-dependent protein stabilisation of hDlg in epithelial cells.

HPV-16 positive CaSKi and HPV-18 positive HeLa, plus HPV-negative HaCaT and C33I cells were grown to 25% (L), 50% (M) or 75% (H) confluence and either treated (+) or not (-) with the proteasome inhibitor CBZ for four hours prior to protein extraction. Western blot was then performed to analyse hDlg protein levels. Actin staining was also performed to confirm equal loading.
Also shown are direct microscope photographs of the cell lines analysed, to compare their morphologies.

Cell type	% of colony formation
HeLa	72 ± 8
CaSKi	0-3
C33I	48 ± 8
HaCaT	0
BRK	0
BRK transformed (E7+ras)	65 ± 10
G	0
AT-1	0-5
MML	60 ± 10

Table 5. Anchorage-independent growth of epithelial cells.

For each experiment, $1x10^5$ 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.

organisation of cell junctions. No changes in the hDlg pattern were however observed either in HeLa or in C33I cells upon increased cell density.

To investigate whether hDlg was indeed being degraded by the proteasome in isolated cells, while being stabilised at sites of cell-cell contact, immunofluorescence staining and confocal laser microscopy were performed, using anti-hDlg monoclonal antibody and FITC-conjugated secondary antibody. As shown in Figure 20, HaCaT skin keratinocytes stained for hDlg protein show a weak, diffused pattern of expression in isolated cells, while 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, while there is a minimal effect on the amount of hDlg protein being expressed at sites of cell contact. In contrast, analysis of the 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, and indeed these cells do not appear to form stable cell junctions. 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 grouped. These results demonstrate that in epithelial cells hDlg is stabilised at sites of cell-cell contact. However, any hDlg that is not localised to cell junctions is rapidly degraded by the proteasome.

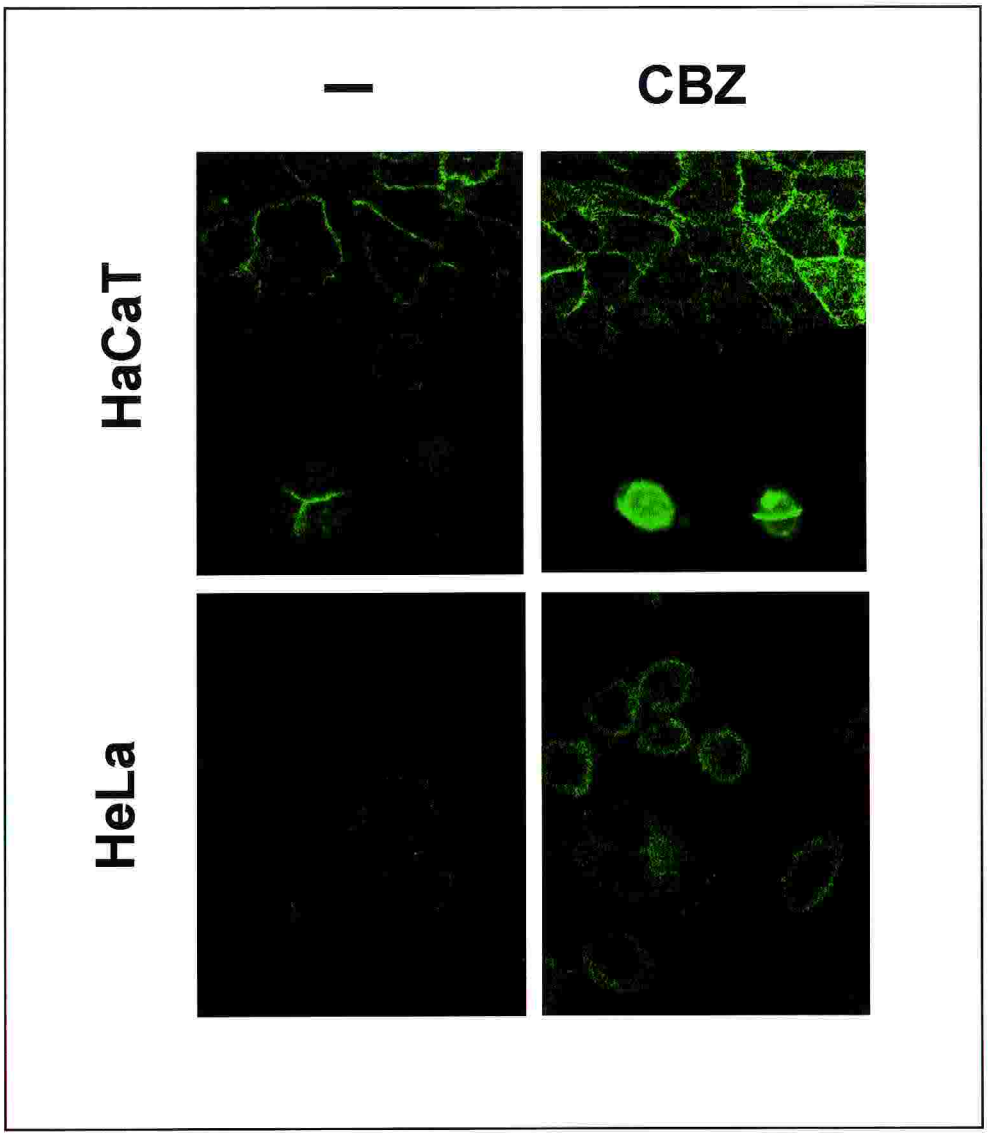


Figure 20. Localisation of hDlg at cell junctions of epithelial cells inhibits its proteasome degradation.

HaCaT skin keratinocytes and HeLa cervical carcinoma cells were either treated or not with CBZ proteasome inhibitor for four hours 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.

Phosphorylation of hDlg.

On Western blots of epithelial cells, the anti-Dlg antibody often detected several closely spaced bands, which could represent different hDlg isoforms produced by differential splicing, as well as postranslationally modified forms of the protein. The analysis of the *hDlg* cDNA has in fact revealed two regions of alternative splicing, which give rise to a high number of combinations of inserted regions, and Northern blots and RT-PCR reveal several *hDlg* mRNA isoforms (Lue et al., 1994; McLaughlin et al., 2002). We have shown here that, upon hDlg protein stabilisation in those cell lines which retain an epithelial morphology, the hDlg protein profile on Western blots also changes, with the appearance of slower migrating forms of the protein. This result indicates that these forms are most likely being degraded at a higher rate by the ubiquitin-proteasome pathway. As previously discussed, signal-dependent modification of cellular proteins by phosphorylation is a common pathway to target specific substrates for ubiquitin-mediated degradation (Laney and Hochstrasser, 1999, for review). Therefore, in order to determine whether the higher molecular weight species of hDlg were indeed phosphorylated forms, proteasome inhibition was performed on CaSKi and HaCaT cells, and the cell extracts were then incubated with λ phosphatase, prior to analysing the pattern of hDlg by SDS-PAGE and Western blot. As shown in Figure 21, in both cell lines the phosphatase treatment dramatically changes the mobility of the higher molecular weight forms of hDlg, demonstrating that they are indeed phosphorylated forms of the protein.

These results indicate that the less stable forms of hDlg protein are phosphorylated, suggesting that this modification might render hDlg more susceptible to ubiquitin-mediated degradation.

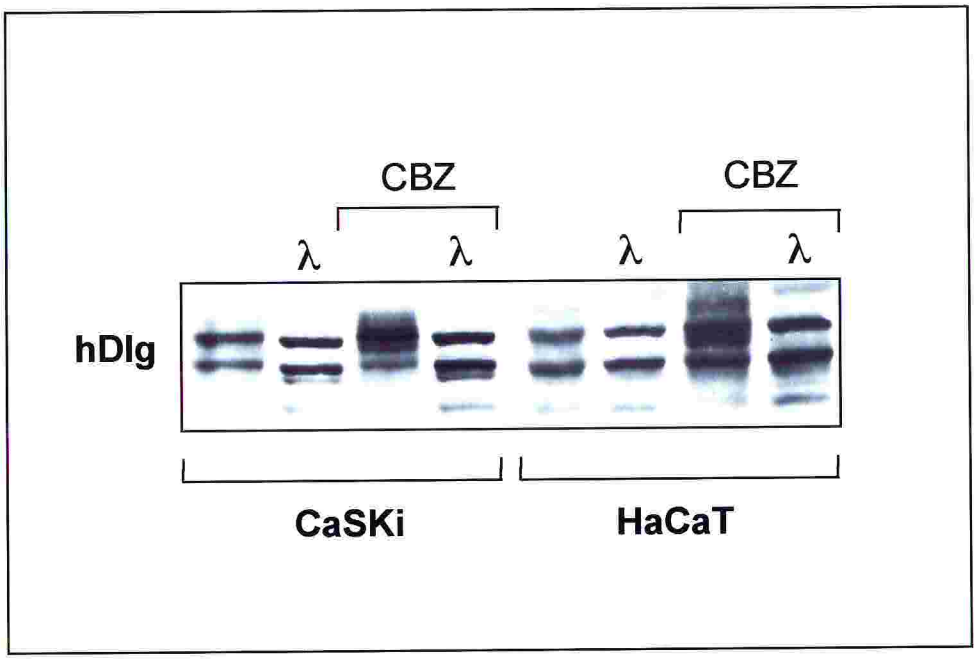


Figure 21. Accumulation of hyper-phosphorylated hDlg protein following proteasome inhibition.

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.

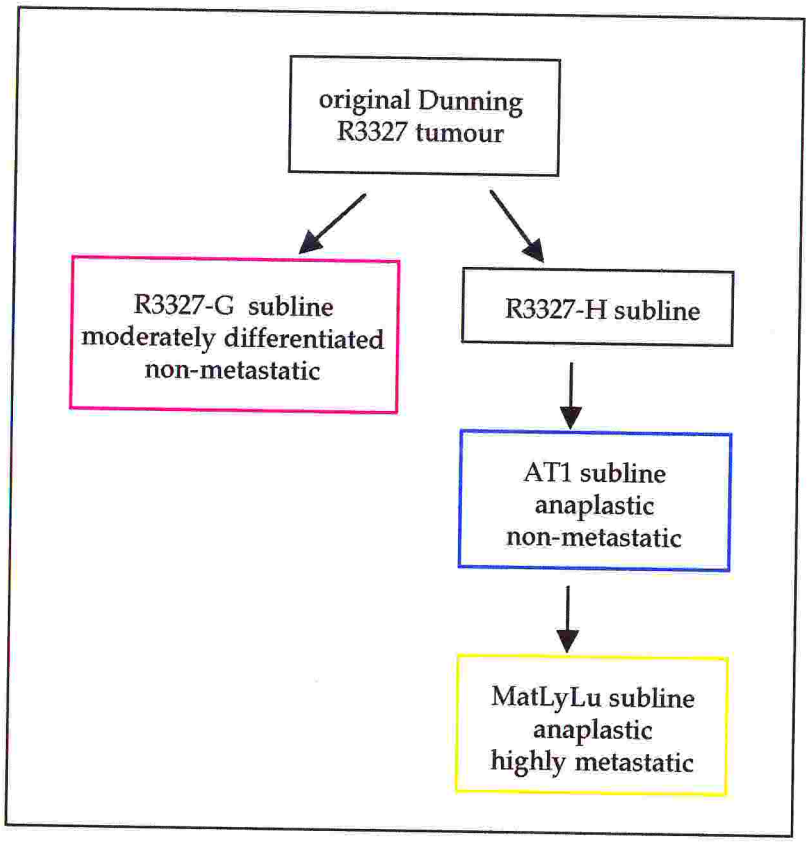
Loss of expression and cell contact-dependent stabilisation of Dlg during malignant transformation.

Based on the above results it would appear that the ability to regulate hDlg protein levels is perturbed as cells lose their epithelial morphology with the ability to form cell junctions and display a more transformed phenotype. To directly investigate this aspect, two different cellular systems were employed.

First, the pattern of Dlg expression was compared between primary rodent epithelial cells and fully transformed cells derived from the identical lineage. Primary baby rat kidney (BRK) cells were prepared from 9 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 by co-expression of 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 that they have also acquired anchorage-independent growth, being able to form colonies in soft agar while their normal counterparts are not (Table 5). As can be seen in Figure 22A, in primary BRK cells the Dlg protein is unstable when the cell density is very low, while its stability increases as the cells become more confluent. In contrast, the amount of Dlg protein does not increase at high cell density in the transformed cells and, notably, very little stabilisation of Dlg follows proteasome inhibition.

We then analysed a second model of epithelial tumour progression, the well characterised and widely used Dunning system of rat prostate cancers (Tennant et al., 2000, for review). This system consists of numerous tumour sublines, which have been derived *in vivo* by serial passaging of a spontaneous rat papillary adenocarcinoma of the prostate called R3327. Three cell lines derived from the parental tumour that can be passaged *in vitro* were chosen for this study. The R3327-G subline is moderately differentiated, and the cells grow at a low rate, forming

epithelial-like sheets and tight intercellular contacts. In contrast the AT1 and MatLyLu sublines are anaplastic, cultured cells have a higher proliferation rate compared with G cells, grow disorderly and make very few and loose cell-cell contacts. Herebelow is a schematic diagram representing the origin and characteristics of the three cell lines.



These cell lines are at well defined stages of malignant progression, as can be seen from the results of the soft agar assays reported in Table 5. While the highly metastatic MatLyLu cells were able to proliferate in both 0.5% and 1% agar, neither G nor AT1 cells showed this ability, consistent with the observation that they are not metastatic in syngeneic rats.

To investigate the levels of Dlg protein and its proteolytic regulation, all three cell lines were grown at 25% (L) and 75% (H) confluence, and cell extracts were then analysed by Western blot. As can be seen in Figure 22B, G cells contain the highest levels of Dlg protein, whose stability is regulated through proteasome degradation at low cell density, and increase consistently at higher cell density. Compared with G cells, AT1 cells contain much lower levels of Dlg protein, due in part to a higher rate of degradation, as indicated by the efficient stabilisation of Dlg observed upon proteasome inhibition. In addition, Dlg protein levels do not significantly increase upon cell contact. Finally, the amount of Dlg protein detected in the MatLyLu cells is extremely low compared with their less transformed counterparts, in part due to degradation through the proteasome pathway, and again no significant stabilisation is seen at high cell density.

Taken together, these results demonstrate that when comparing cells derived from the same lineage but at different levels of neoplastic transformation, the expression of Dlg is lower as the cells become more highly transformed. In the case of the prostate model this appears to depend, at least in part, on a higher rate of degradation by the proteasome pathway. In addition, the ability to stabilise Dlg in response to increased cell contact is completely lost as cells become fully transformed, suggesting that loss of Dlg regulation is a key event during the progression of malignancy. These results provide strong support to the hypothesis that Dlg is a tumour suppressor in mammalian cells.

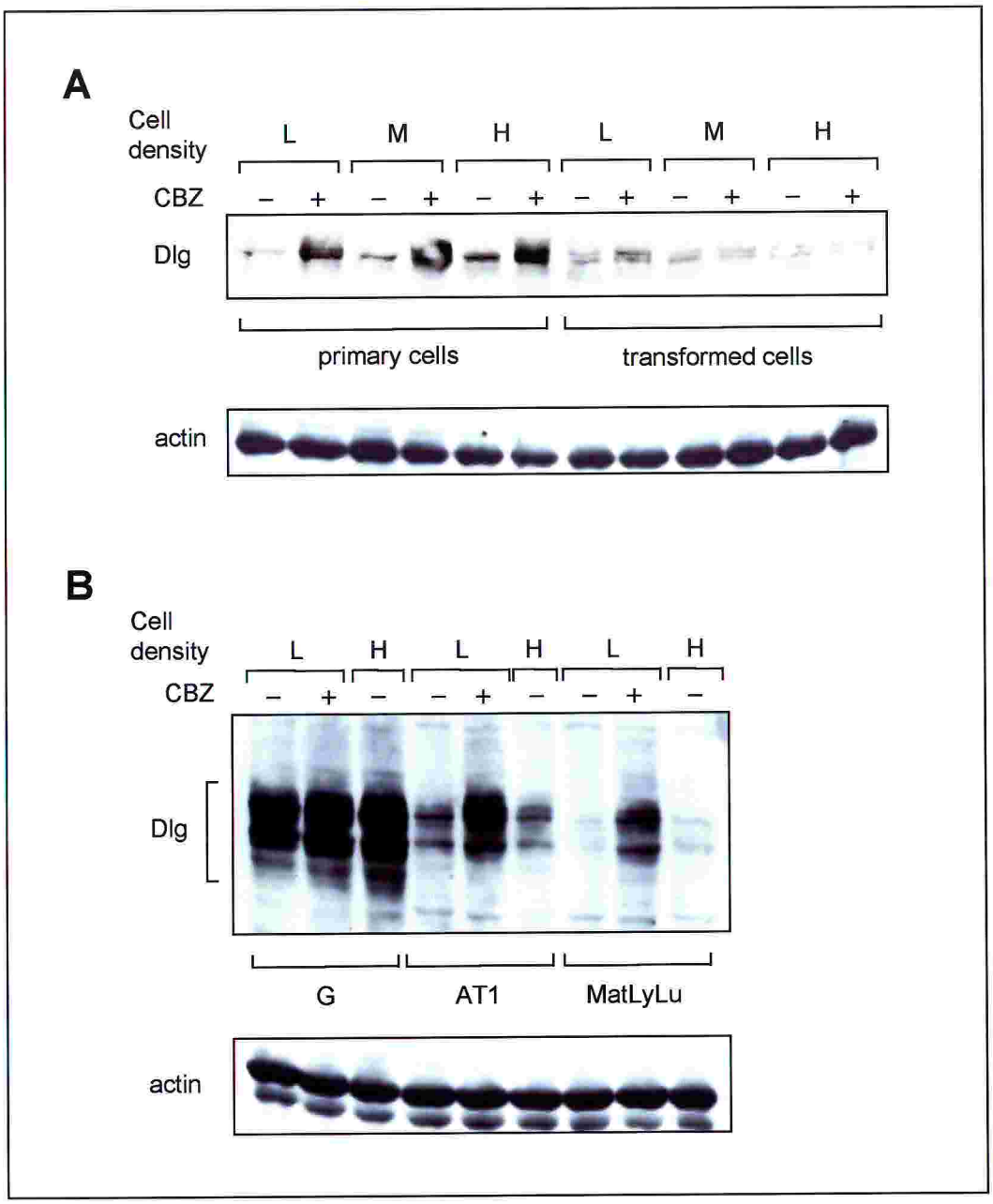


Figure 22. Consequences of cell transformation on expression and density-dependent stabilisation of Dlg protein.

(A) Primary baby rat kidney (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. Actin staining was also performed to confirm equal loading.

(B) Three different sublines of Dunning R3327 rat prostate cancer: G (moderately differentiated, non metastatic), AT1 (anaplastic, non metastatic) and MatLyLu (anaplastic, highly metastatic) were grown to 25% (L) or 75% (H) confluence, and either treated or not with the proteasome inhibitor CBZ for two hours. The Dlg protein pattern was then analysed by Western blot. Actin staining was also performed to confirm equal loading.

hDlg sequence contains a putative consensus binding-site for the F-box protein β-TrCP.

Having demonstrated that hDlg is regulated by the proteasome in both E6-dependent and –independent pathways, it was then of interest to investigate the mechanisms underlying this regulation in the cell. It has been reported that β-catenin exists in multimolecular complexes together with the APC tumour suppressor protein, axin, the GSK3β kinase and the F-box protein β-TrCP (Kitagawa et al., 1999; Liu et al., 1999). These associations serve to regulate the activity of β-catenin, through stimulating its ubiquitination by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex, and thereby its degradation by cytoplasmic proteasomes (Hart et al., 1999, and references therein). It has also been demonstrated that, at the membrane-cytoplasm interface of epithelial cells, hDlg forms a complex with APC, which also contains β-catenin (Matsumine et al., 1996). These findings prompted us to investigate the possibility that the hDlg protein could also be turned over through a pathway similar to β-catenin. It had been reported that the F-box protein β-TrCP, which is the substrate-binding component of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex, selectively binds to its substrates through recognising a serine-phosphorylated consensus sequence: DSGXXS (in single-letter code, where X is any residue). Strikingly, an analysis of the primary sequence of Dlg protein revealed a sequence motif perfectly matching this consensus: DSGLPS involving residues 596-601, which lie in the SH3 domain of Dlg. Interestingly, this sequence motif appears to be conserved among the human and rat Dlg proteins. Figure 23A shows a comparison of the putative β-TrCP consensus binding-site of rat and human Dlg with those of some known substrates of β-TrCP, such as β-catenin and IκB.

Dlg interacts with the F-box protein β-TrCP in vitro.

This finding prompted us to investigate if the hDlg and β-TrCP proteins are indeed able to interact. GST-fused full-length Dlg protein together with a series of deletion

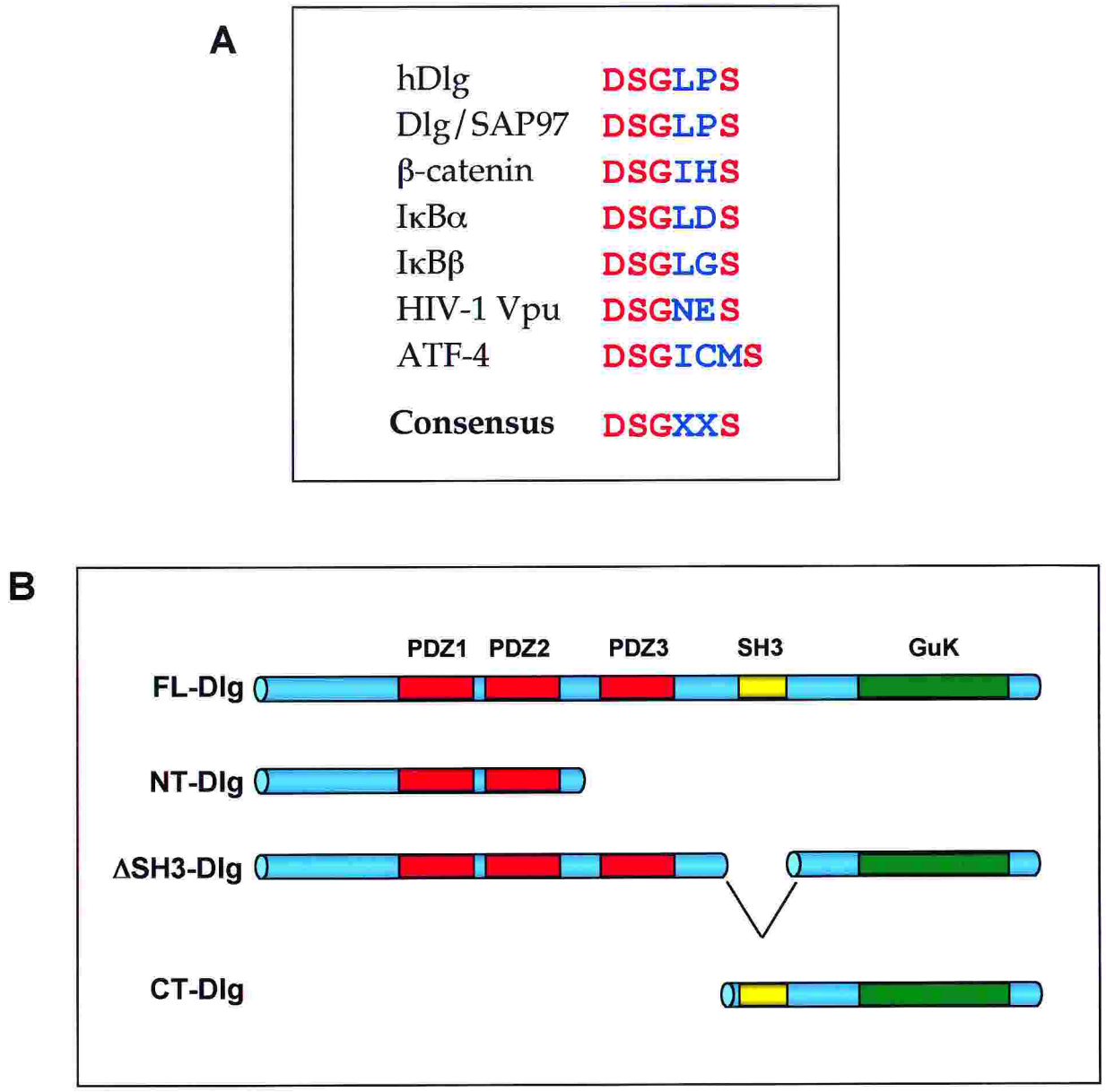


Figure 23.

(A) Comparison of the six-aminoacid consensus motif identified in hDlg and rat Dlg/Sap97 with those bound by β-TrCP in β-catenin, IκB, HIV Vpu and ATF4 proteins. **(B)** Schematic representation of the full-length and deletion mutant Dlg proteins used in this study. NT-Dlg contains the N-terminal domain plus the first two PDZ domains (aa 1-382). ΔSH3-Dlg has a deletion encompassing the SH3 domain (aa 549-617). CT-Dlg contains the SH3 and GuK domains (aa 539-911).

mutants were produced in bacteria, purified and analysed in a pull-down assay with *in vitro* translated β-TrCP protein. The different Dlg proteins are shown schematically in figure 23B. These are: the full-length protein FL-Dlg, the mutant ΔSH3-Dlg lacking the SH3 domain, the mutant NT-Dlg containing Dlg N-terminal domain plus the PDZ domains 1 and 2, and the mutant CT-Dlg containing the SH3 and GuK domains of Dlg. As can be seen from Figure 24A, β-TrCP did not show any significant interaction neither with FL-Dlg nor with any of the mutant proteins in a standard *in vitro* binding assay. This was not too surprising however, since it has been shown that β-TrCP binds selectively to phosphorylated substrates, and indeed it has also been shown that β-catenin needs to be phosphorylated in order for β-TrCP to bind *in vitro* (Hart et al., 1999). Therefore the GST-fusion proteins were first incubated with ATP plus 10 μg of HaCaT total cell extract as a source of cellular kinases. One set of reactions was performed in the presence of ^{32}P-γATP, to account for phosphate incorporation (Figure 24B), and a second set of reactions where done without radiolabel in order to assess binding of β-TrCP. The autoradiogram shown in Figure 24B demonstrates that both Dlg and its mutant derivatives are all phosphorylated by cellular kinases present within the cell lysate. When the pull-down assay was then performed following incubation of the beads with the cell extract, both the FL-Dlg and CT-Dlg proteins, but not the mutants lacking the SH3 domain, showed greatly enhanced levels of interaction with the β-TrCP protein (Figure 24A). To determine whether phosphorylation of Dlg by cellular kinases was indeed responsible for β-TrCP binding, GST-Dlg proteins were first incubated with HaCaT cell extract and then either treated or not with λ phosphatase before testing them for binding to β-TrCP. As can be seen in Figure 24C, phosphatase treatment almost abolished the binding of β-TrCP to FL-Dlg, while no difference was observed in the background level of interaction with the ΔS-Dlg mutant protein. In conclusion, these results show that the Dlg and β-TrCP proteins interact *in vitro*, and that the SH3 domain of Dlg is necessary for the interaction, while the N-terminus and the first two PDZ domains are not involved. Moreover, phosphorylation by an as yet unidentified cellular kinase(s) is necessary in order to allow the purified Dlg protein to interact with β-TrCP.

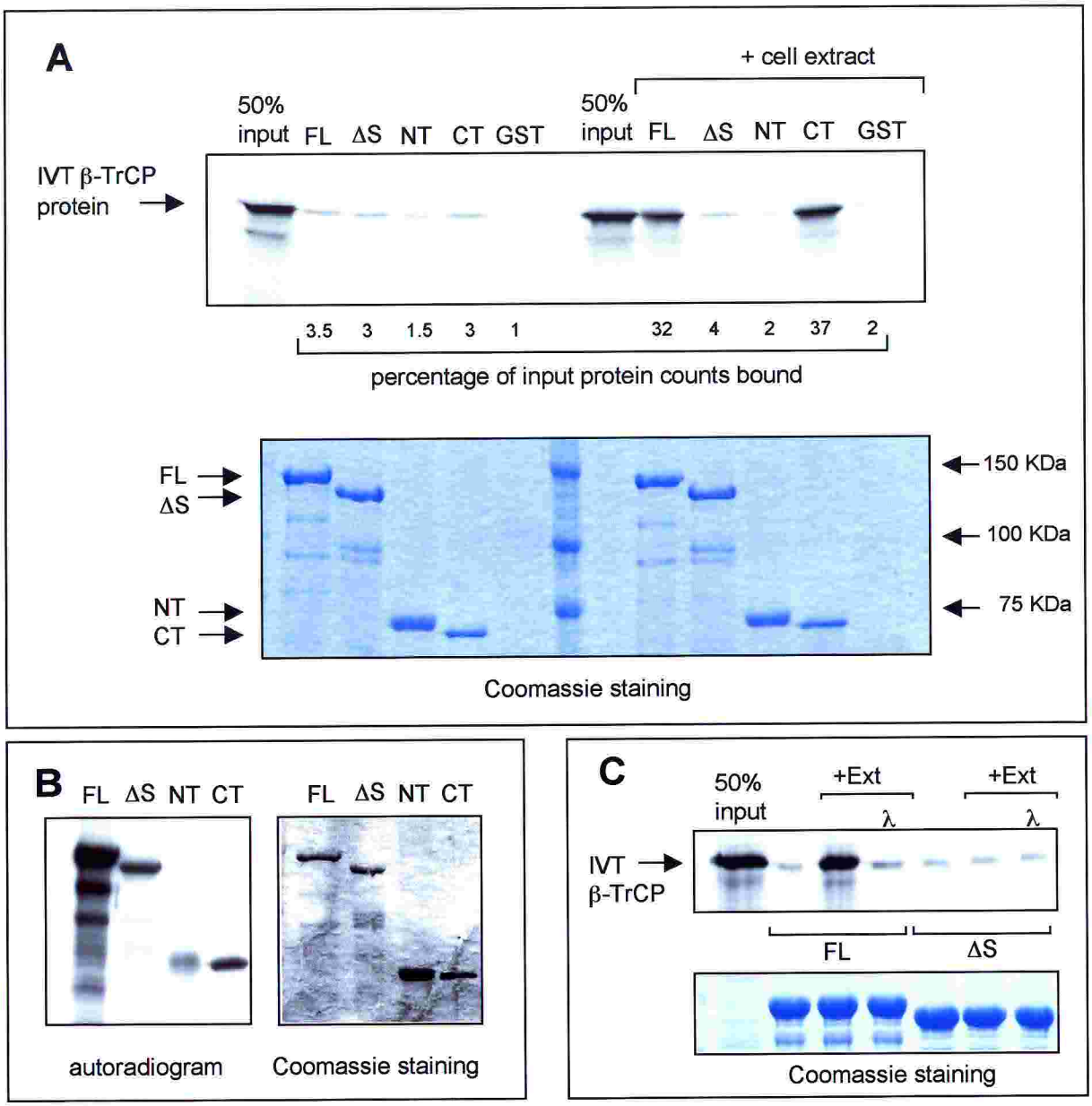


Figure 24. Binding to β-TrCP protein *in vitro* is mediated by the SH3 domain of Dlg and is dependent upon its phosphorylation by cellular kinases.

(A) Radiolabelled *in vitro* translated (IVT) β-TrCP protein was incubated with purified GST-Dlg fusion proteins (FL, ΔS, NT and CT) or with GST alone as a control for 1 hour at RT. The same experiment was performed in parallel incubating the GST-fusion proteins with 10 μg of total extract of HaCaT cells before mixing them with IVT β-TrCP. After washing, bound proteins were assessed by SDS-PAGE and autoradiography. The panel below represents Coomassie stained gel showing fusion protein levels.
(B) GST-fused Dlg proteins were incubated *in vitro* with 10 μg of total cell extract and with 5 μCi of ^{32}P-γATP and subsequently analysed by SDS-PAGE and autoradiography. The panel beside shows Coomassie staining of the gel.
(C) Wild-type (GST-FL) and mutant (GST-ΔS) Dlg proteins were either left untreated or treated with HaCaT cell extract and ATP as in (A) and either subsequently treated or not with λ phosphatase. Pull down assays were then performed as in (A) with *in vitro* translated, radiolabelled β-TrCP protein.

Dlg and β-TrCP proteins interact in vivo.

To investigate whether the Dlg and β-TrCP proteins are also able to interact *in vivo*, 293 cells were transiently transfected with Myc-tagged β-TrCP and either HA-tagged FL-Dlg or mutant ΔSH3-Dlg expression vectors. At 36 hours post-transfection, the cells were treated for 2 hours with the proteasome inhibitor CBZ, then harvested and immunoprecipitated with either anti-HA or anti-Myc antibodies. To avoid interference by the immunoglobulin heavy chains that are expected to migrate just below the β-TrCP protein on SDS-PAGE, all the antibodies were previously crosslinked to the sepharose resin (see Materials and Methods). As expected, when the FL-Dlg was transfected, the polyclonal anti-HA antibody immunoprecipitated two bands of approximately 100 KDa, while two bands of slightly lower molecular mass were immunoprecipitated from cells transfected with the ΔSH3-Dlg expression plasmid (Figure 25A). Interestingly, when β-TrCP and FL-Dlg were cotransfected, the anti-HA antibody also precipitated a protein of about 60 KDa, which corresponds to β-TrCP, since this also comigrates with the β-TrCP protein immunoprecipitated by the anti-Myc antibody. Moreover, this band was absent from the anti-Myc immune complex when β-TrCP was not transfected. In addition, two protein bands corresponding to FL-Dlg were precipitated by the anti-Myc antibody when β-TrCP and FL-Dlg were cotransfected, but not when only FL-Dlg was present. These results clearly show that FL-Dlg associates with β-TrCP when both are overexpressed in 293 cells. In agreement with the results obtained from the *in vitro* binding assay, ΔSH3-Dlg did not coimmunoprecipitate with β-TrCP, clearly showing that the SH3 domain of Dlg is necessary for the interaction of the two proteins *in vivo*.

From the above *in vitro* binding assay it appears that β-TrCP binds to phosphorylated Dlg. In order to investigate this possibility further, the anti-Myc immunoprecipitate was treated with λ phosphatase prior to gel loading. As can be seen from Figure 25A (track 3) the mobility of the coimmunoprecipitated HA-Dlg protein changed dramatically. In fact upon phosphatase treatment this comigrated exactly with the lower molecular mass form of HA-Dlg found in the anti-HA immunoprecipitate.

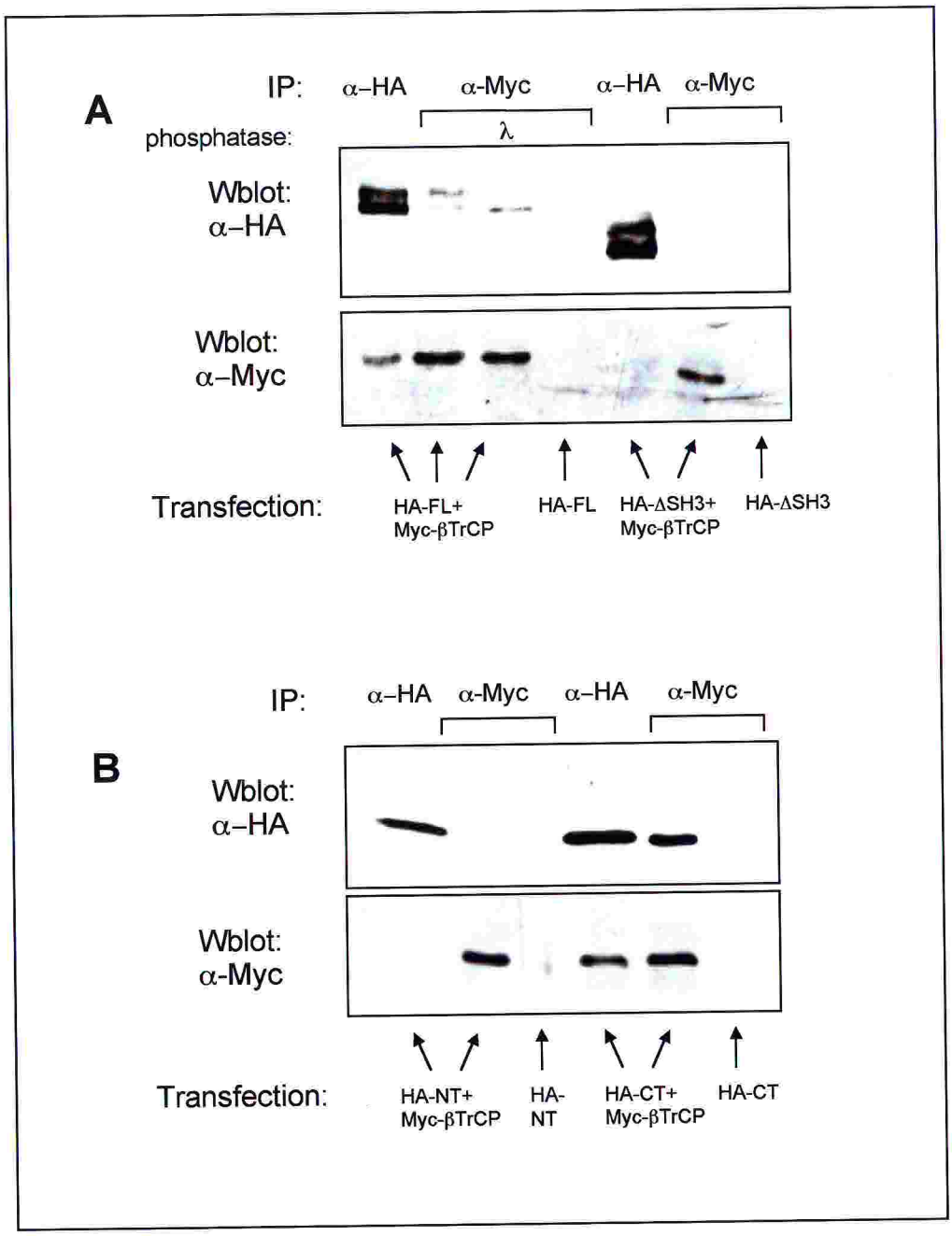


Figure 25. β-TrCP associates *in vivo* with phosphorylated Dlg protein and the interaction is dependent on the presence of the SH3 domain of Dlg.

(A) The HA-FL Dlg protein and its deletion derivatives HA-ΔSH3 and **(B)** HA-NT and HA-CT were transiently expressed in 293 cells, either alone or together with Myc-tagged β-TrCP protein. After 36 hours the cells were treated with CBZ proteasome inhibitor for 2 hours and then harvested. Cell lysates were immunoprecipitated with either anti-HA or anti-Myc antibodies, and subsequently analysed for the presence of HA-tagged Dlg and Myc-tagged β-TrCP proteins by Western blot. λ= treatment of the immunoprecipitate with λ phosphatase before gel loading.

These results clearly demonstrate that the form of the Dlg protein that interacts with β-TrCP is phosphorylated. This result, together with the observation that the SH3 domain is essential for the interaction, is consistent with the hypothesis that β-TrCP will form a complex with Dlg when this is phosphorylated within its SH3 domain.
This analysis was then extended to the other Dlg mutants, and Myc-tagged β-TrCP was transfected together with expression vectors encoding either HA-tagged CT-Dlg, which contains the SH3 domain, or NT-Dlg, which is devoid of this domain. The protein interactions were then examined by coimmunoprecipitation as above. As can be seen from Figure 25B, CT-Dlg was precipitated together with β-TrCP, and *vice versa*, when the two proteins were coexpressed. In contrast the NT-Dlg did not show any interaction with the F-box protein. These results are in agreement with those observed *in vitro*, and they further enforce the hypothesis that the SH3 domain is required for mediating the interaction between Dlg and β-TrCP.

Endogenous hDlg and β-TrCP proteins interact in HaCaT keratinocytes.

Having demonstrated that Dlg and β-TrCP could interact when overexpressed in 293 cells, it was then obviously important to investigate the interaction between the endogenous hDlg and β-TrCP proteins. To do this immunoprecipitations of untransfected HaCaT cell lysates were performed using a polyclonal anti-Dlg antiserum, and the resulting immunoprecipitates were then probed with anti-β-TrCP antibodies on Western blot. The experiment was also performed by first immunoprecipitating the lysates with anti-β-TrCP antibodies and subsequently probing for the presence of hDlg by Western blot. As shown in Figure 26, several protein bands migrating at about 100-130 KDa and recognised by the anti-Dlg antibody were present in the anti-β-TrCP immunoprecipitate. These bands were identifiable as forms of hDlg since they were also found in the anti-Dlg immunoprecipitate, and they were not seen when the cell lysates were precipitated with a non-immune serum (PI). In addition, treatment with λ phosphatase changed the mobility of the hDlg protein bands immunoprecipitated by the anti-β-TrCP

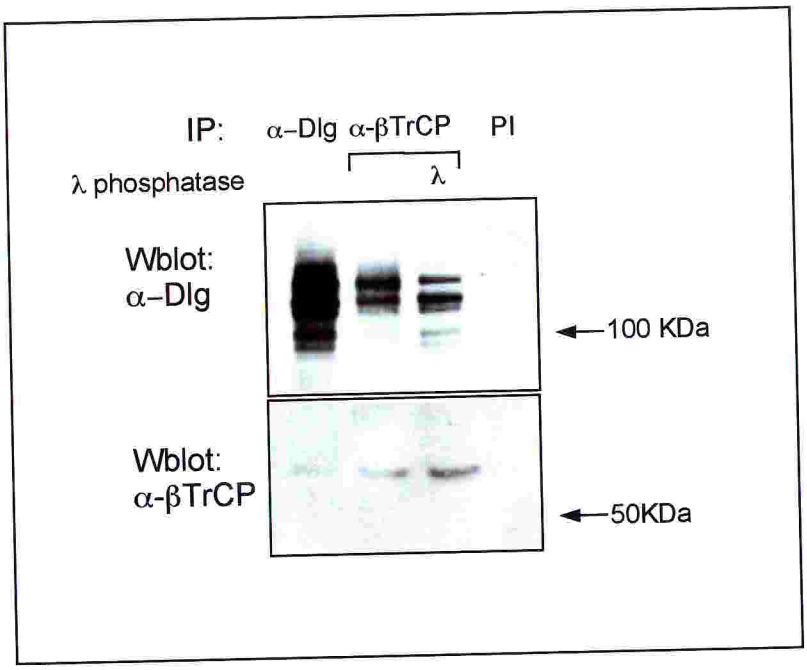


Figure 26. Association between endogenous hDlg and β-TrCP proteins in HaCaT keratinocytes.

Untransfected HaCaT keratinocytes were treated for 2 hours with CBZ proteasome inhibitor before harvesting. Cell lysates were then immunoprecipitated with either anti-Dlg or anti-βTrCP polyclonal antibodies or with non-immune serum as a control. The βTrCP immunoprecipitate was either treated or not with λ phosphatase before gel loading.

antibody. This confirms that the endogenous hDlg protein, which interacts with endogenous β-TrCP protein in HaCaT keratinocytes is also phosphorylated. The hDlg-β-TrCP protein interaction was also detected using the anti-Dlg antiserum to coprecipitate a 60 KDa protein recognised by anti-β-TrCP antibodies, and which comigrates with the β-TrCP protein present in the anti-β-TrCP immunoprecipitate. From these results it can be concluded that endogenous hDlg and β-TrCP proteins are present in the same complex within HaCaT epithelial cells.

Overexpression of wild-type β-TrCP enhances ubiquitin-conjugation of Dlg protein in vivo, while F-box deleted ΔFβ-TrCP inhibits this process.

The finding that hDlg interacts with β-TrCP, the F-box subunit of the $SCF^{\beta TrCP}$ ubiquitin ligase complex, which directs specific cellular substrates to the proteasome pathway induced us to investigate the role of β-TrCP in the ubiquitination and degradation of Dlg. Therefore an *in vivo* ubiquitination assay was established, which could allow detection of the short-lived ubiquitin-intermediates of Dlg. HA-FLDlg was transiently transfected into 293 cells together with a ubiquitin expression vector, either alone or in the presence of wild-type β-TrCP expression plasmid. 24 hours post-transfection, the cells were metabolically labelled with ^{35}S Met/Cys for two hours, either in the presence of CBZ proteasome inhibitor to block degradation of ubiquitin-conjugated proteins or in the presence of DMSO as a control. The cells were then harvested and cell lysates were immunoprecipitated with anti-HA antibody in the presence of CBZ and of the isopeptidase inhibitor N-ethylmaleimide (10 mM) to inhibit destruction of the ubiquitin-conjugates. As can be seen from Figure 27B, proteasome inhibition causes the appearance of a ladder of high-molecular weight HA-Dlg forms, which are not visible in the absence of CBZ. Coexpression of wild-type β-TrCP does very slightly affect the amount of nonconjugated Dlg protein, however, upon proteasome inhibition the appearance of high molecular weight forms of Dlg is highly enhanced in β-TrCP transfected cells with respect to the control experiment where β-TrCP was omitted. This result suggests that the high molecular

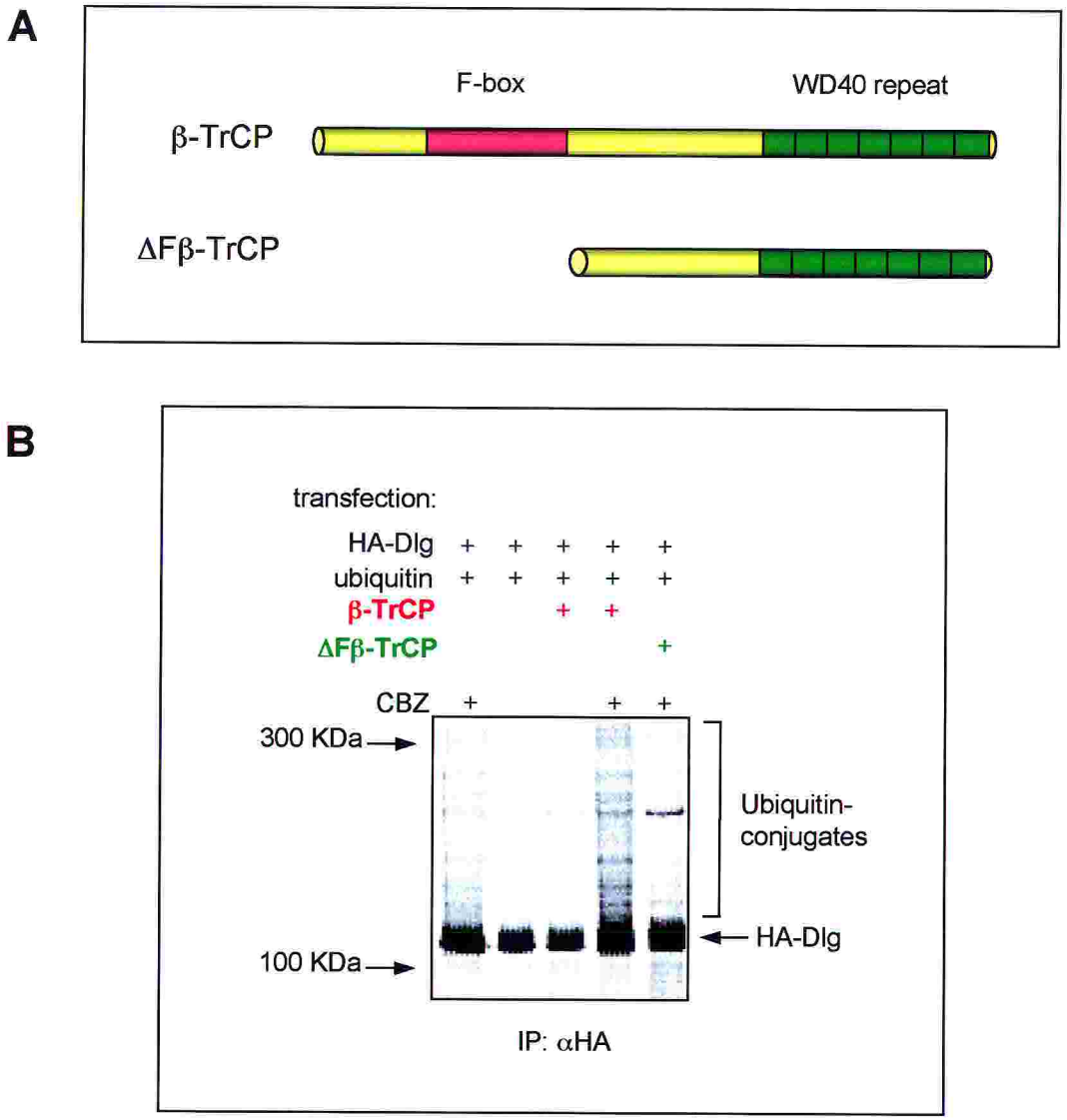


Figure 27. Dlg ubiquitination *in vivo* is enhanced by the presence of wild type β–TrCP.

(A) Schematic representation of the wild type β–TrCP and of the F-box deleted ΔFβ–TrCP proteins used in this study.
(B) 293 cells were transfected with HA-FLDlg expression plasmid, together with ubiquitin expression plasmid and with either wild type β–TrCP or F-box deleted mutant ΔFβ–TrCP expression vector, or with empty plasmid as a control. After 24 hours the cells were metabolically labeled for 2 hours with ^{35}S Met/Cys either in the presence of CBZ proteasome inhibitor or not. HA-Dlg protein was recovered by immunoprecipitation with anti-HA antibody and visualised by SDS-PAGE and autoradiography.

weight forms of Dlg, which are stabilised by proteasome inhibition, might correspond to ubiquitin-conjugated forms of the protein. To confirm this supposition, we coexpressed HA-FLDlg together with ubiquitin and with a mutant β-TrCP protein lacking the F-box domain, ΔFβ-TrCP. ΔFβ-TrCP is unable to bind to Skp1, and therefore acts as a dominant negative inhibiting the ubiquitination of the cellular substrates of β-TrCP (Margottin et al., 1998). The wild type and mutant β-TrCP expression vectors are schematically represented in Figure 27A. As can be seen in the last track of Figure 27B, when Dlg was coexpressed together with ΔFβ-TrCP and the cells were then treated with the proteasome inhibitor CBZ, only a very weak ladder of high molecular weight forms of Dlg was visible, in comparison to the clear ladder seen in the cells expressing wt β-TrCP protein, as well as with control-transfected cells. This result suggests that ubiquitination of Dlg *in vivo* is inhibited by the dominant-negative mutant ΔFβ-TrCP protein, and this further demonstrates that endogenous β-TrCP protein has a role in the ubiquitination of Dlg *in vivo*.

Expression of wild-type β-TrCP but not of mutant ΔFβ-TrCP causes proteasome-mediated degradation of full-length Dlg protein in vivo.

The above results suggest that β-TrCP is responsible for the ubiquitin-conjugation of Dlg *in vivo*, and therefore it was logical to determine if it can also cause a reduction in the Dlg steady state levels within the cell. To do this 2 μg of HA-tagged FLDlg expression plasmid together with increasing amounts of β-TrCP expression vector were transfected into 293 cells. 36 hours after transfection, the cells were either treated with CBZ proteasome inhibitor or with DMSO as a control, and the steady-state levels of Dlg were then analysed by Western blot with anti-HA antibody. The results obtained are shown in Figure 28. The lower panel shows probing of the Western blots for β galactosidase expression, confirming equal levels of transfection efficiency. As shown in Figure 28A, expression of β-TrCP caused a marked reduction in the steady-state levels of Dlg protein. This effect was dose-dependent and could be completely rescued by proteasome inhibition. To further confirm these results, the same

experiment was done in the presence of ΔFβ-TrCP and this time no reduction in the levels of Dlg protein was obtained.

The analysis was then repeated on the Dlg deletion mutants. Figure 28A shows that β-TrCP has no effect on the steady-state levels of ΔSH3-Dlg mutant, which lacks the SH3 domain and is unable to bind β-TrCP. The slight variation in the ΔSH3-Dlg levels seen in the third lane is very likely to be non specific and rather due to a relatively low transfection efficiency, as confirmed by concomitant lower expression of the β galactosidase protein in the panel below. Interestingly however, proteasome inhibition also leads to an increase in the levels of ΔSH3-Dlg, even though this protein does not appear to be degraded through the β-TrCP pathway. This result suggests the existence of ubiquitin ligases, different from β-TrCP, which mediate proteasome degradation of ΔSH3-Dlg protein *in vivo.*

The same analysis was then performed on the other two mutants, CT-Dlg and NT-Dlg (Figure 28B). Unexpectedly, the levels of the CT-Dlg mutant protein were totally unaffected by the expression of β-TrCP. This was somewhat surprising, since this mutant protein is able to bind β-TrCP *in vitro* and *in vivo* with high affinity. Therefore this result would suggest that the binding of β-TrCP to Dlg alone is not sufficient to trigger the proteasome-mediated degradation of Dlg, and suggests that N-terminal sequences are also required for this process to occur. On the other hand, the NT-Dlg protein was also completely unaffected by β-TrCP expression, however this was not unexpected since no binding to β-TrCP has been shown for this protein.

Taken together, the above results allow us to conclude that β-TrCP is responsible for downregulation of Dlg protein levels through the proteasome pathway. Moreover, we can conclude that the SH3 domain of Dlg, which mediates the binding to β-TrCP, is necessary for ubiquitin-mediated degradation by β-TrCP, however other sequences in the N-terminal part of Dlg also appear to be required for efficient degradation. Finally, it would appear that other ligases also exist which are able to regulate the turnover of Dlg.

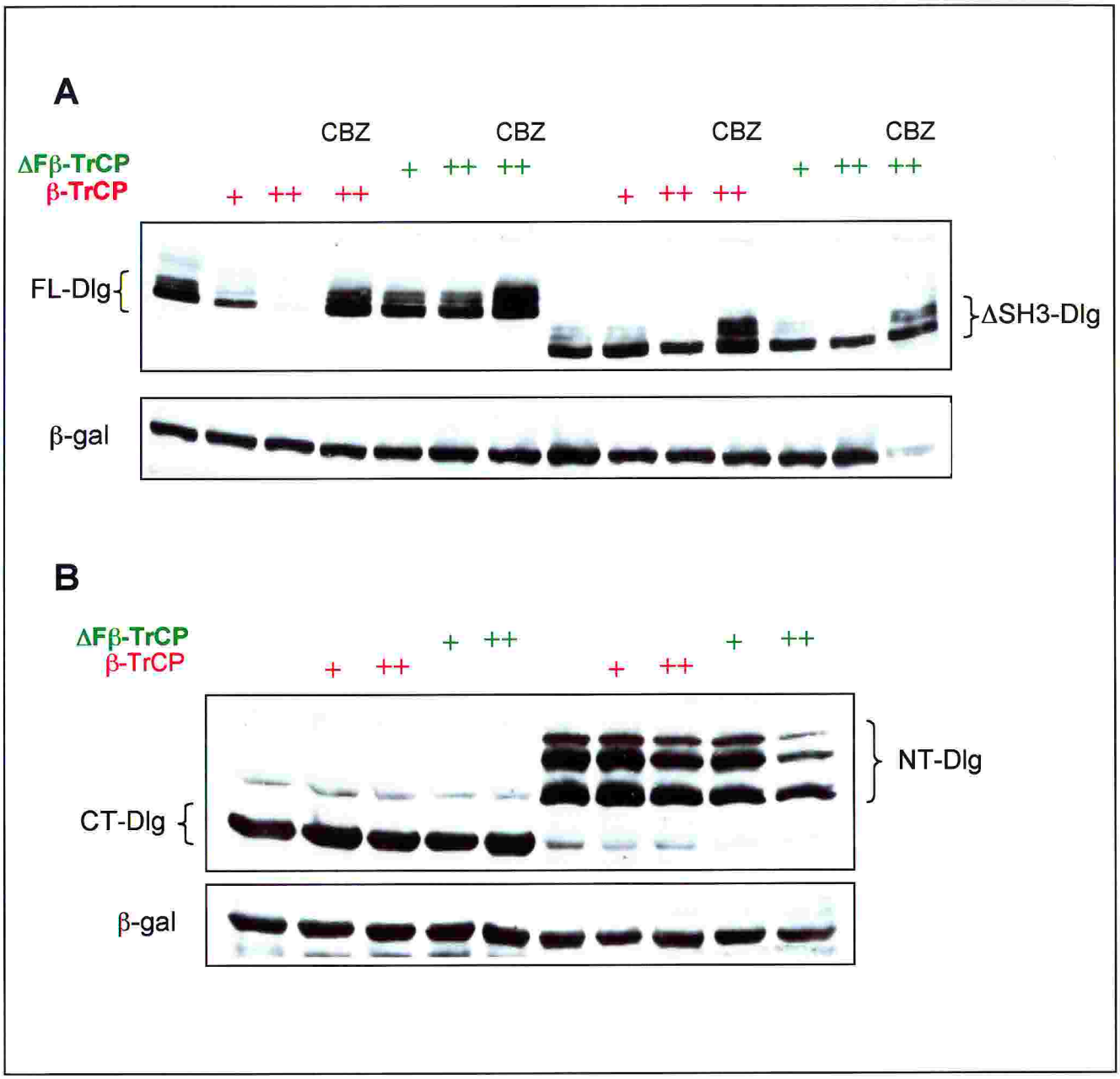


Figure 28. β-TrCP overexpression causes proteasome-mediated degradation of full-length Dlg, but not of ΔSH3-, NT- and CT- Dlg deletion mutant proteins.

HA-tagged FL-Dlg, ΔSH3-Dlg **(A)**, CT-Dlg and NT-Dlg proteins **(B)** were transiently overexpressed in 293 cells either alone or in the presence of wild type β-TrCP or mutant ΔFβ-TrCP proteins. At 36 hours post transfection cells were either treated with the proteasome inhibitor CBZ or with DMSO as a control **(A)** or were left untreated **(B)**. The remaining HA-Dlg proteins were then assessed by Western blot with anti-HA antibodies. The lower panels show staining of the Western blots with anti β-galactosidase antibody to confirm equal levels of transfection efficiency.

Wild-type β-TrCP, but not mutant ΔFβ-TrCP can decrease the stability of full-length Dlg protein in vivo.

In order to verify that the decrease in the steady-state levels of Dlg caused by β-TrCP overexpression was due to decreased stability of Dlg protein, a series of pulse-chase experiments was done on the HA-FLDlg protein in 293 cells, either in the absence or presence of coexpressed β-TrCP or ΔFβ-TrCP proteins. 24 hours post transfection the cells were labelled with ^{35}S Met-Cys for one hour and then either harvested (time zero) or chased for 3, 6 or 9 hours. The remaining HA-Dlg protein was then immunoprecipitated from the cell lysates with anti-HA antibody, separated on SDS-PAGE and analysed by PhosphoImager scanning. Three independent experiments were performed, the mean values of the counts calculated, and finally Dlg stability was measured plotting the amount of protein remaining as a function of time: the amount of FLDlg at time zero was defined as 100%. Figure 29 shows the result of a typical pulse-chase experiment and the graph below represents the mean results of three separate experiments. As can be seen, HA-Dlg is a relatively unstable protein, with a half-life of approximately 4.5 hours. Coexpression of β-TrCP accelerates Dlg turnover, shortening its half-life up to 2.6 hours. Upon coexpression of the transdominant-negative ΔFβ-TrCP protein, the stability of Dlg is slightly increased, with an estimated half-life of about 5.3 hours.

In order to verify that the effects of β-TrCP on Dlg protein stability were specific, the same experiment was repeated using ΔSH3Dlg and CTDlg, which have been shown previously to be unaffected by expression of β-TrCP. 293 cells were transfected with HA-ΔSH3Dlg expression vector, either together with β-TrCP expression plasmid or with vector alone, then the cells were metabolically labeled and chased as above. As can be seen from Figure 30A, the ΔSH3Dlg protein is slightly more stable if compared with the full-length Dlg protein, its half-life being of roughly 5.5 hours. This observation is consistent with the assumption that this protein cannot be targeted for degradation by endogenous β-TrCP. Similarly, overexpression of β-TrCP does not significantly alter the turnover of ΔSH3Dlg, and this confirms the above results. The

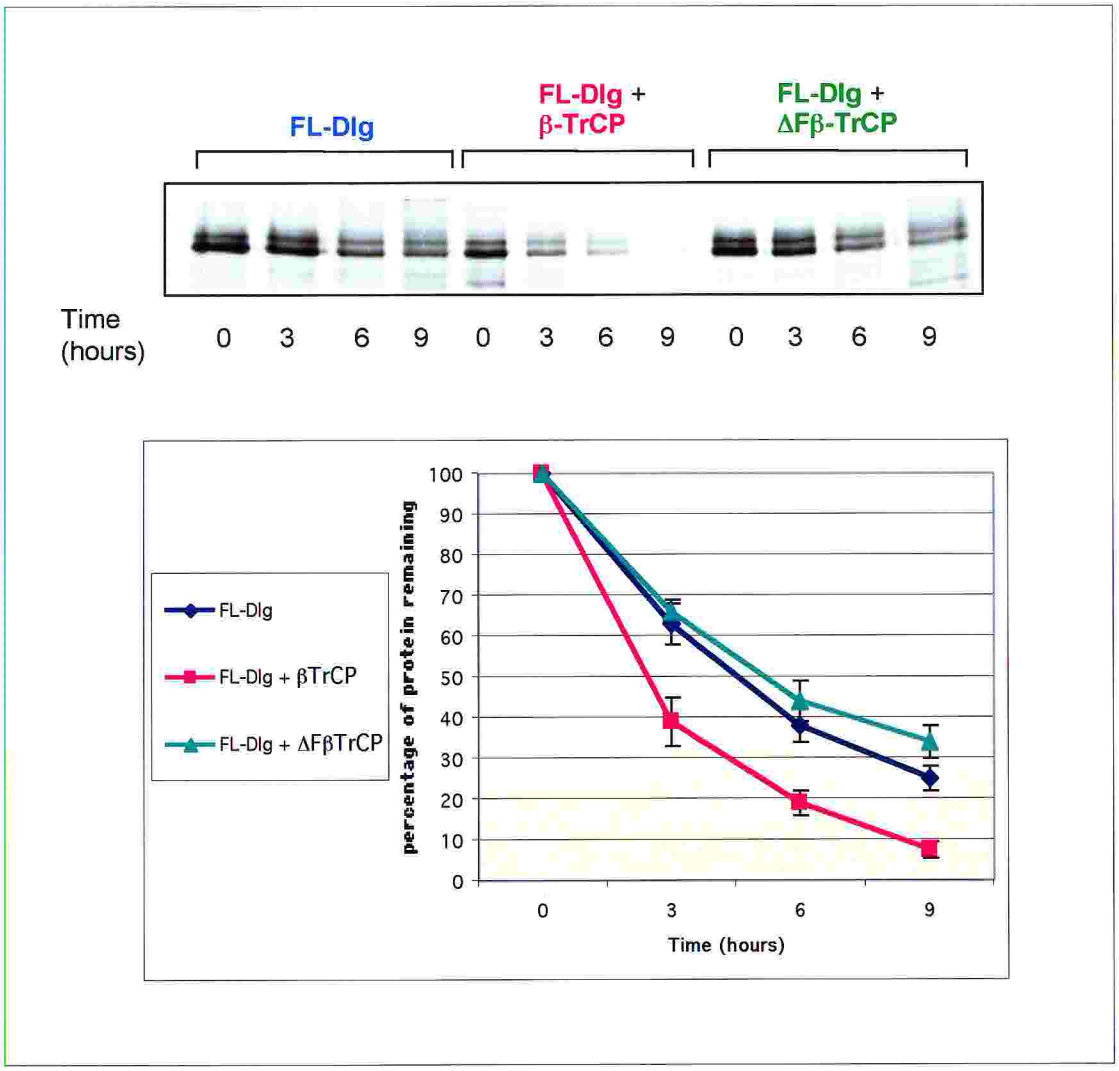


Figure 2 9. Wild-type but not F-box deleted β-TrCP decreases the stability of Dlg protein *in vivo*.

Shown is a pulse-chase analysis of the turnover rate of HA-tagged FL-Dlg protein in 293 cells. HA-FLDlg was either cotransfected with empty pCDNA vector (blue line) or with wild type β-TrCP (red line) or mutant ΔFβ-TrCP (green line) expression vectors. 24 hours post transfection the cells were metabolically labelled for 1 hour with ^{35}S Met/Cys and then chased for the times indicated. Remaining Dlg protein was immunoprecipitated via the HA tag and then assessed by SDS-PAGE and autoradiography. Quantisation of the autoradiograms was performed by PhosphoImager and the amount of protein at 0 hours was defined as 100%. Results obtained from three independent experiments are shown in the graph below, where the percentage of Dlg protein remaining is plotted as a function of time.

stability of the HA-CTDlg mutant protein was also measured both in the presence and absence of β-TrCP. Figure 30B shows that HA-CTDlg protein also has a longer half-life when compared with the full-length Dlg protein. Moreover, only a slight variation in the half-life of HA-CTDlg is observed as a consequence of β-TrCP overexpression. These results further confirm those obtained above, suggesting that the ability to interact with β-TrCP protein is alone not sufficient for triggering proteasome-mediated degradation of the HA-CTDlg mutant protein.

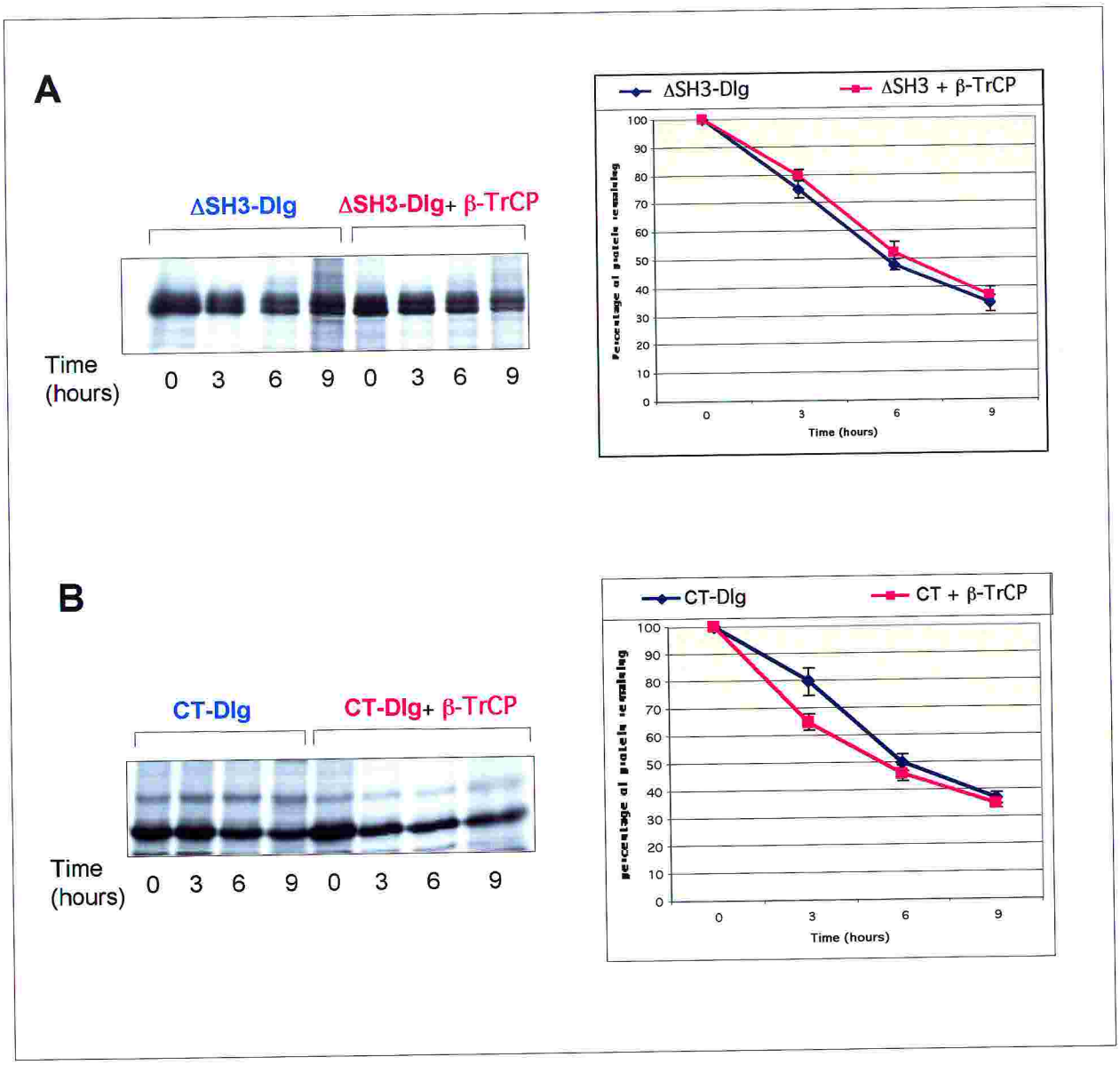


Figure 30. Stability of ΔSH3-Dlg and CT-Dlg mutant proteins is not affected by overexpression of β-TrCP.

HA-ΔSH3Dlg **(A)** and HA-CTDlg **(B)** mutant proteins were expressed in 293 cells either alone or in the presence of wild type β-TrCP protein. The cells were radiolabelled for 1 hour with ^{35}S Met/Cys and then chased for the times indicated. Remaining Dlg proteins were immunoprecipitated via the HA tag and then assessed by SDS-PAGE and autoradiography. Quantitation of the autoradiograms was performed by PhosphoImager and the amount of protein at 0 hours was defined as 100%. Results obtained from two separate sets of experiments are shown in the graphs.

DISCUSSION

Proteasome-mediated degradation of the hDlg tumour suppressor protein in epithelial cells.

Several proteins involved in cell contact and adhesion are either the product of proto-oncogenes or tumour suppressor genes: the disorganisation of epithelial junctions can indeed 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 been recently established by the finding that a group of *Drosophila* tumour suppressors acts in concert to regulate both cell growth and polarity (Bilder and Perrimon, 2000; Bilder et al., 2000). Their products, Dlg, Lgl and Scrib are membrane-associated proteins, which depend on each other for the correct localisation and formation of epithelial junctions. Mutations in any of these genes cause very similar phenotypes, with aberrantly shaped cells, alterations in apicobasal polarity and overproliferation of epithelial cells. Defective cell-cell adhesion could in fact impair growth control by compromising contact inhibition. Dlg is a multidomain protein that can organise the clustering of receptors, channels and signalling complexes at the cell membranes: mislocalisation of growth factor receptors or signalling molecules would clearly affect cell proliferation. The modes of action of these oncosuppressors are likely to be conserved also 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 the translocation of hDlg from cytoplasmic pools to the plasma membrane (Reuver and Garner, 1998), where it interacts with the cytoskeletal protein 4.1 (Lue et al., 1994) and with hCASK (Nix et al., 2000; Lee et al., 2002), both of which play central roles in the formation of cell-cell junctions. In addition, hDlg forms a complex with the APC tumour suppressor protein (Matsumine et al., 1996), thereby inhibiting cell cycle progression (Ishidate et al., 2000). The central role of hDlg and 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 indeed the expression of high-risk HPV E6 has been shown to disrupt epithelial tight junctions (Nakagawa and Huibregtse, 2000). As already discussed, HPVs infect keratinocyte stem cells, 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 the pathways regulating cell differentiation and proliferation in order to allow for 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 part of the thesis, the regulation of the hDlg tumour suppressor protein has been studied in epithelial cells, as a means of beginning to understand how the HPV E6 proteins can alter the cellular pathways regulated by hDlg in favour of viral replication, as well as contributing to malignancy.

It is clear that hDlg is degraded by the proteasome pathway in cell lines derived from cervical tumours containing high-risk HPV oncogenes. However, a considerable variability in the basal levels of hDlg protein as well as in the extent of stabilisation achieved upon proteasome inhibition was observed in cell lines containing HPV-18. These data could reflect differences in the hDlg gene expression levels as well as variability in the rate of hDlg degradation. This could depend either on differences in the abundance of the E6 protein that is triggering destruction of hDlg, or on cellular components of the ubiquitin pathway. It seems unlikely, however, that the weak effects of the proteasome inhibitors on the accumulation of hDlg in HeLa cells will reflect a low responsiveness of the cells to these compounds, since p53 protein was efficiently stabilised in HeLa cells following proteasome inhibition, once it was induced by DNA damage (see Part 1). Interestingly, the results presented also provide evidence that the 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. Indeed, previous evidence had already suggested that ubiquitin mediated degradation of hDlg can occur even in the absence of HPV E6 expression. The studies presented here also show that readily detectable levels of hDlg protein are found in those cells retaining an epithelial morphology, such as HaCaT keratinocytes, CaSKi and C4-I cervical cancer cell lines, primary BRK cells and the R3327-G rat prostate cancer cell line. All these cells are characterised by their ability to establish high numbers of intercellular junctions, which hold the cells together in structured epithelial layers and regulate their growth upon cell contact. Conversely, very low levels of hDlg protein were found in those cells that exhibit a highly transformed phenotype, have mesenchimal morphology, make very few and loose intercellular contacts and grow in a disorderly fashion without contact inhibition. These are, for example, Hela and C33I cervical cancer cell lines, fully transformed BRK cells and the anaplastic prostate carcinoma AT1 and MatLyLu cell lines. Thus, loss of hDlg expression correlates with a more transformed phenotype, and from our analysis this would appear to occur, at least in part, at the level of mRNA expression, while in part it depends on a high rate of protein degradation. It should be noted that inhibition of proteasome activity has a strong effect on the stabilisation of hDlg protein where its basal levels are relatively high, while in those cells that contain very low amounts of hDlg, the effects of proteasome inhibition are less apparent.

Using both Western blotting and immunofluorescence analysis, it was also possible to show that the levels of hDlg protein are regulated by the degree of cell contact. Thus, in isolated epithelial cells hDlg is mainly cytoplasmic and it is continually degraded through the proteasome pathway. However, once contacts with neighbouring cells are established the hDlg protein that localises at membrane junctions becomes stabilised. Since these cell types grow in islands, where only the cells at the periphery are thought to divide, it is important to note that in HaCaT cells the level of hDlg expression does not vary between cells which are in G0 or G1 and cells which are progressing towards mitosis. Nonetheless, the subcellular localisation of hDlg

changes during the cell cycle (P. Massimi personal communication). Again, density-dependent stabilisation is only seen in the less transformed, epithelial-like cell types, such as HaCaT and CaSKi cell lines, and appears to be independent of the presence of HPV sequences. In contrast those cells, which have lost their ability to establish intercellular contacts such as HeLa and C33I cells, also appear to be unable of stabilising hDlg protein at high cell densities. Interestingly, the analysis of primary BRK cells highlights a very similar pattern of Dlg regulation. Once those cells become fully transformed their ability to regulate Dlg stability is lost together with the capacity to form cell junctions, and the protein fails to accumulate even when the cells reach high density. Analysis of three different sublines of the Dunning R3327 rat prostate carcinoma also provided similar results. Only the most differentiated G cell line, which grows orderly in structured monolayers, showed a clear ability to stabilise Dlg protein as a result of increasing intercellular contacts. In contrast, the two anaplastic cell lines AT1 and MatLyLu, which are more transformed and grow in the absence of stable intercellular contacts, have much lower levels of Dlg expression and are also incapable of stabilising Dlg protein at high cell density. These results suggest that loss of hDlg expression, and in particular of the ability to upregulate its levels upon cell contact, is a vital step during malignant progression. Indeed, by performing a series of soft agar assays, it was observed that in those cell types having an impaired regulation of Dlg, there is a parallel acquisition of an invasive phenotype with the 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. Indeed, the ability to target hDlg for degradation has an important role in the transforming potential of the high-risk HPV E6 oncoproteins (Kiyono et al., 1997). It will therefore be of great general interest to determine if loss of hDlg expression is a common feature of all highly malignant tumours, or at the very least, of those of epithelial origin.

Not all the forms of the hDlg protein that can be discriminated by SDS-PAGE are degraded with equal efficiency, with some of them being stabilised only after sustained treatment with proteasome inhibitors. By treatment of cell extracts with

phosphatase, it was possible to show that the less stable forms of hDlg are phosphorylated. This suggested that phosphorylation could be part of a signalling cascade that regulates proteasome degradation of hDlg, as has been frequently reported, for example for the cellular targets of SCF ubiquitin ligase complexes (Laney and Hochstrasser, 1999). On the other hand, a high degree of cell-cell contact also results in the appearance of phosphorylated forms of hDlg that are absent in isolated cells, and this would imply their direct involvement in the molecular organisation of the epithelial junctions. Posttranslational modifications have been shown to regulate the membrane localisation of several members of the Dlg family. Palmitoylation controls the association of PSD-95 with the plasma membrane (Topinka and Bredt, 1998; El-Husseini et al., 2000), while phosphorylation of insect Dlg by CaMKII kinase regulates its anchoring to the synaptic complex (Koh et al., 1999). Moreover, phosphorylation of the PDZ-protein ZO-1 has been proposed to play a role in the assembly of tight junctions (Kurihara et al., 1995). Phosphorylation of Dlg has been reported previously: in human T lymphocytes it was found to interact with the $p56^{lck}$ tyrosine kinase (Hanada et al., 1997), and it was also reported to be phosphorylated at mitosis in HeLa cells, possibly by a PDZ-binding kinase (Gaudet et al., 2000). In addition, analysis of Dlg sequence reveals a multitude of putative consensus sites for several cellular kinases.

Therefore it would appear that the hDlg protein is subjected to a very complex pattern of regulation, which also involves its modification by phosphorylation. It could be speculated that some phosphorylations are either the requisite or the consequence of its participation in the construction of cell junctions. On the other hand, phosphorylated hDlg which is not localised at membrane sites is targeted for rapid degradation. Much work will be required in order to understand the cellular pathways that regulate hDlg, however from this thesis it can be concluded that this complex regulation is severely disrupted in highly transformed cells.

The F-box protein β-TrCP associates with phosphorylated Dlg and stimulates its degradation through the proteasome pathway.

The biological functions, intracellular localisation and cellular binding partners of the hDlg and β-catenin proteins have several aspects in common, and this suggested that the two proteins could be regulated through similar pathways.

The functional roles of β-catenin have been extensively studied. A membrane pool is involved in cell-cell adhesion: it links cadherins to α-catenin and the actin cytoskeleton, which results in the formation of the adherens junctions (Peifer, 1995; Gumbiner, 1997). Similarly, hDlg has also been shown to interact with the actin cytoskeleton and to colocalise with E-cadherin at adherens junctions (Reuver and Garner, 1998). A soluble cytoplasmic and nuclear pool of β-catenin has a role in Wnt signalling that promotes cell proliferation in embryonic and adult tissues (Gumbiner, 1995, for review), and β-catenin activity is tightly controlled by means of regulating its stability. It is now well established that a multiprotein "destruction complex" containing APC, Axin, the GSK3β kinase and the F-box protein β-TrCP determines β-catenin degradation through the ubiquitin-proteasome pathway in the absence of Wnt signaling (Kitagawa et al., 1999; reviewed in Peifer and Polakis, 2000). Similarly in this thesis it was shown that while hDlg is stabilised when localised at sites of cell-cell contact, free hDlg protein is rapidly removed from the cytoplasm by proteasome-mediated degradation. This finding supports the idea of different hDlg pools performing specific roles in the cell that need to be tightly regulated. Consistent with this, it has recently been reported that different Dlg isoforms have specific subcellular localisation, the I3 insertion targeting Dlg to the membrane while the I2 insertion determines its nuclear localisation (McLaughlin et al., 2002).

Analysis of the hDlg sequence revealed a conserved six-residue motif homologous to a sequence found in β-catenin. Once it is phosphorylated by GSK3β, this motif mediates the interaction of β-catenin with the F-box protein β-TrCP, leading to its ubiquitination by the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase complex (Hart et al., 1999; Winston et al., 1999; Latres et al., 1999). As already mentioned, a similar motif mediates the

binding of β-TrCP to other protein substrates such as IκB and ATF4. Notably, we have demonstrated that hyperphosphorylated forms of hDlg are degraded through the proteasome pathway.

It was therefore logical to investigate whether Dlg was also subject to regulation by β-TrCP. In a series of *in vitro* binding assays it was found that, although purified Dlg protein is unable to interact with β-TrCP, it nonetheless acquires this ability following phosphorylation by a cellular kinase(s). Moreover, the SH3 domain of Dlg containing the putative β-TrCP-binding consensus sequence was found to be necessary for the interaction. Complex formation between Dlg and β-TrCP *in vivo* was then confirmed in transfected epithelial cells, and the SH3 domain was again shown to be required. In addition it was also found that the Dlg protein that interacts with β-TrCP is hyperphosphorylated. The simplest explanation for these results would be that β-TrCP binds to its phosphorylated recognition site within the SH3 domain of Dlg. However, to directly prove this hypothesis it will be necessary to specifically mutate the residues within the putative consensus binding-site, and then analyse the ability of the mutant Dlg protein to interact with β-TrCP.

It was also possible to show that the association between the endogenous hDlg and β-TrCP proteins does indeed occur in HaCaT keratinocytes, when both proteins are present at their physiological levels and at their normal cellular locations. Based on these observations it was tempting to suggest that β-TrCP may have a role in the regulation of Dlg turnover. Indeed, upon coexpression of β-TrCP and Dlg a significant increase in the amount of ubiquitin-conjugated Dlg intermediates was observed. Interestingly, a mutant β-TrCP protein deleted for its F-box domain, ΔFβ-TrCP, inhibited the ubiquitination of Dlg. It has been reported that the F-box domain is necessary for anchoring β-TrCP to Skp1, the core component of the ligase complex (Margottin et al., 1998). ΔFβ-TrCP protein therefore is unable to bind to Skp1, however it can still interact with its substrates through the C-terminal WD repeats. In this way ΔFβ-TrCP is thought to function as a dominant negative, which sequesters the substrates of wild type β-TrCP without targeting them for ubiquitination. Indeed, ΔFβ-TrCP has been reported in several cases to act as a dominant negative inhibiting

the ubiquitination and proteasome degradation of the β-TrCP substrates β-catenin, IκBα, ATF4 and Vpu/CD4 (Spencer et al., 1999; Kitagawa et al., 1999; Lassot et al., 2001; Margottin et al., 1998). The fact that ΔFβ-TrCP inhibits ubiquitin-conjugation of Dlg *in vivo* strongly suggests a role for the endogenous β-TrCP protein in this process. Our supposition was further confirmed by the demonstration that β-TrCP is able to reduce both the steady-state levels and the stability of Dlg protein *in vivo*. The presence of the SH3 domain was also found to be required for β-TrCP-induced degradation of Dlg through the proteasome pathway, consistent with the binding data. Strikingly however, the CT-Dlg deletion mutant lacking the N-terminus and the PDZ domains, although still able to interact with β-TrCP, was not targeted for degradation. This result implies that complex formation with the F-box protein β-TrCP is not alone sufficient for inducing the proteolytic destruction of Dlg. It is possible that the lysine residues, which accept the ubiquitin moieties lie within the N-terminal sequences of Dlg, or alternatively that these N-terminal domains are necessary to mediate the binding with other cellular proteins required for the ubiquitination of Dlg. Interestingly the NT-Dlg mutant protein, which contains the N-terminus together with the first two PDZ domains of Dlg, has previously been reported to be ubiquitinated and degraded by the proteasome *in vivo* (Gardiol et al., 1999; Gardiol et al., 2002). Moreover, a short Dlg fragment containing the second PDZ domain alone has also been found to be degraded through the proteasome pathway, when stably expressed in U2OS cells (Gardiol et al., 2002). Two alternative situations can be proposed in order to explain these results. If the $SCF^{\beta\text{-}TrCP}$ complex is the sole ubiquitin ligase responsible for regulating Dlg stability, it could be argued that the Dlg protein truncations, which contain the PDZ2 motif, are nonetheless able to interact with APC through this domain (Matsumine et al., 1996). By means of binding to APC they would then indirectly associate with the multiprotein complex which also contains the $SCF^{\beta TrCP}$ ubiquitin ligase, and thereby be non-specifically ubiquitinated. It should be remembered that exogenously overexpressed proteins are far from their physiological concentrations, and this might result in forcing the protein interactions to occur under non-specific conditions. An alternative explanation

is that a second ubiquitin ligase also exists, whose binding site lies within the second PDZ domain of Dlg. This hypothesis would be consistent with the observation that the ΔSH3 mutant, which cannot be recognised by the β-TrCP protein, is nonetheless degraded by the proteasome pathway, not only when transiently transfected but also upon stable expression (Gardiol et al., 2002). It could be speculated that the two ligases are responsible for regulating the stability of two separate pools of Dlg protein, which play different functions within the cell. It has been frequently proposed that different isoforms of Dlg protein may play different roles, and recently it has been reported that, while the I3 insertion downstream of the SH3 domain determines its association with the cortical cytoskeleton through binding to the 4.1 protein (Marfatia et al., 1996), the alternatively spliced I2 domain is responsible for nuclear localisation of hDlg (McLaughlin et al., 2002). By analogy with its *Drosophila* homolog, the mammalian Dlg protein is likely to govern both polarity and proliferation of epithelial cells, and it is possible that the nuclear and membrane pools of Dlg perform these distinct functions in the cell. Comparative analysis of the protein interactions of the different Dlg molecules will be extremely important for understanding their specific functional roles. In particular, it will be interesting to determine whether the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase targets one or more specific hDlg isoforms. Unfortunately, it has been observed that upon transient overexpression the subcellular localisation of the I2 and I3-containing isoforms of Dlg cannot be differentiated (P. Massimi personal communication). Consistent with this, preliminary results would also indicate that β-TrCP is able to bind and degrade both Dlg isoforms when these are overexpressed. Discrimination of the endogenous hDlg proteins by use of isoform-specific antibodies would therefore appear necessary in order to understand whether β-TrCP is specifically targeting one or both isoforms.

At this point, although many pieces are still missing from the puzzle, a tentative model for the regulation of hDlg in epithelial cells can be hypothesised. hDlg is a MAGUK protein, which contains different protein-protein interaction domains such as PDZ, SH3 and GuK, which allow it to interact with several protein partners

(McLaughlin, 2002, and references therein). Thus, when hDlg is recruited to the adherens junctions, these domains are likely to be engaged by other membrane-associated partners. Interestingly, the MAGUK protein hCASK has been identified as a binding partner for the SH3 domain of hDlg (Nix et al., 2000). This leads to the intriguing possibility that masking the SH3 domain by either hCASK or an as yet unidentified component of cell junctions could prevent its recognition by a specific cellular kinase. In contrast, when hDlg is not engaged at cell junctions its SH3 domain would be unmasked and therefore available to be phosphorylated and subsequently recognised by β-TrCP, leading to degradation of cytoplasmic hDlg. But why should a rapid degradation of soluble hDlg be required? By analogy with other short-lived proteins it is tempting to speculate that the free hDlg protein might be involved in the regulation of cell signaling, and tight control of its accumulation would therefore be required for fine tuning the signal. Indeed, complex formation between hDlg and APC has been reported to cause growth inhibition, by negatively regulating cell cycle progression from the G0/G1 to S phase of the cell cycle (Ishidate et al., 2000). The fact that it can complex with both APC and β-TrCP could lead to the speculation that hDlg is involved in β-catenin degradation, thereby inhibiting Wnt signalling, however this does not seem to be the case (Ishidate et al., 2000).

In conclusion, the roles of hDlg in the cellular pathways regulating cell growth and polarity are still far from being elucidated, however the number of reports describing new cellular partners of hDlg is rapidly growing. Analysis of the changes in hDlg expression and regulation in cell transformation and cancer can be expected to remain a very interesting topic in the forthcoming years.

CONCLUSIONS AND FUTURE PERSPECTIVES.

Tumour progression is a multifactorial and multistep process and those activities of HPV E6 that contribute towards it are a paradigm for carcinogenesis (Figure 31). A cell can become neoplastic as a consequence of genomic mutations resulting in the activation of oncogenes, which drive it towards uncontrolled proliferation, as well as in the inactivation of tumour suppressor genes, which control important cellular checkpoints. Cells have a variety of these mechanisms. These include cell cycle blocks, which protect them from the detrimental effects of replicating a damaged genome and of irregularly distributing their chromosomes among daughter cells, as well as apoptosis that eliminates defective cells, from which malignant clones could originate. Progressive impairment of these safety mechanisms leaves the cell unprotected from the effects of oncogene activation. Amongst the tumour suppressors are also proteins which control cell differentiation pathways as well as being involved in the maintenance of cell-cell contact, cell-matrix adhesion and polarity. Loss of these controls can give a cancer cell a growth advantage, which contributes to its ability to move and metastasise far from its original body site.

During HPV infection, E6 needs to play multiple roles, interfering with several cellular pathways in order to create a favourable environment for viral replication, as well as neutralising the cellular surveillance controls that are turned on as a result of unscheduled DNA synthesis being induced by the E7 oncoprotein. These requirements are more difficult for the high-risk HPV types, which begin to replicate their genomes in differentiating cells, and therefore the activities of the E6 and E7 proteins have more pronounced effects on their cellular target proteins and, occasionally, also on cellular fate. Thus, high-risk HPV E6 blocks apoptosis by targeting p53, Bak and Myc proteins for degradation, however this leaves the cell unprotected from the effects of DNA mutations. This is confirmed by biochemical and epidemiological studies reporting a synergy between HPV oncogenes and chemical carcinogens in the development of malignancy (Song et al., 2000; Daling et al., 1992). Moreover, E6 inhibits terminal differentiation and senescence, thereby contributing to

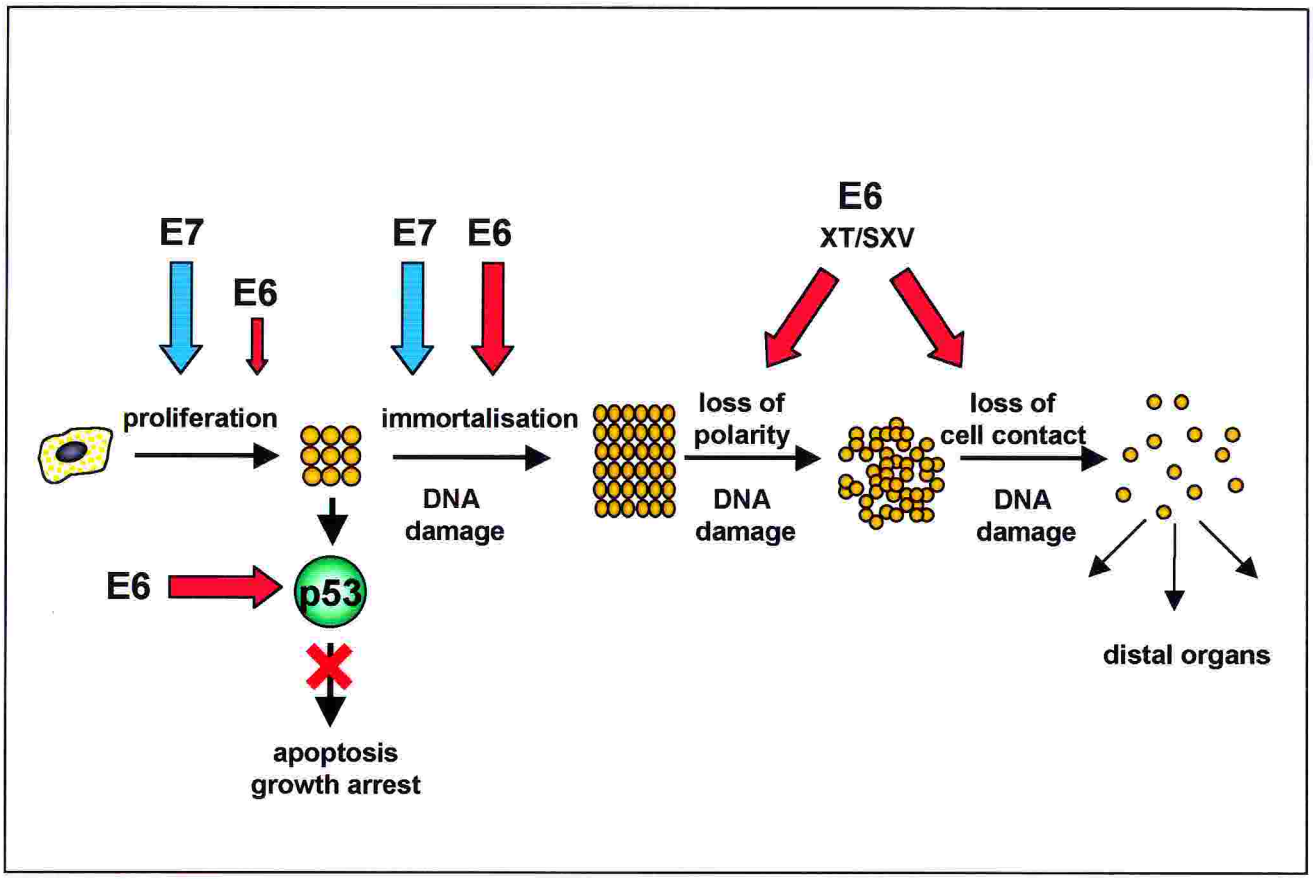


Figure 31. Contribution of high-risk HPV E6 to different stages of tumour progression.

The role of E6 in tumour promotion is weak compared with that of E7, which actively stimulates cell proliferation. In contrast E6 promotes malignant progression: degradation of p53 overcomes growth arrest and apoptosis allowing accumulation of DNA damage, and induction of telomerase contributes towards immortalisation. Finally, targeting PDZ-containing proteins through the C-terminal PDZ-binding motif (XT/SXV) of E6 causes loss of cell polarity and contact, strongly contributing to the malignant phenotype.

cell immortalisation. Finally, by targeting a class of PDZ domain-containing proteins, E6 can also affect cell contact and polarity and, when the cellular pathways regulating these interactions are impaired, either due to mutation or exogenous stimuli, this final loss of control in the immortalised cell can lead to the invasive and metastatic phenotype. It should be clear, however, that the above picture is far from the "physiological" outcome of an HPV infection, representing instead the most unfortunate result of a progressive and multifactorial process that only occurs on some occasions, after the HPV sequences become integrated in the host genome. Nonetheless, cervical cancer is an extremely serious disease, representing the third major cause of cancer-related death in women worldwide (Parkin et al., 1999), and the study of the E6 cellular targets has important implications for the development of effective therapeutic strategies.

In this study three cellular targets of high-risk HPV E6 have been investigated.

Although it is well known that the oncogenic E6 proteins target p53 for proteasome mediated degradation, this does not appear to be the only route by which E6 overcomes its functions. We have demonstrated that inhibition of E6-induced degradation does not always lead to increased p53 levels. In several cervical cancer cell lines p53 can be stabilised only after additional genotoxic insult, indicating a lack of intrinsic activation despite the presence of viral oncogenes. Therefore, although in some cases it would appear feasible to reactivate functional p53 protein by blocking E6-mediated degradation, thus bringing about growth arrest and apoptotic responses in HPV transformed cells, this does not appear to be always applicable. Moreover, we have found that, similarly to other viral oncoproteins, among the strategies used by E6 to inactivate p53 is its cytoplasmic sequestration. We have demonstrated that in HPV-positive cervical cancer cells the nuclear localisation of p53 in response to DNA damage is blocked even if proteasome degradation is inhibited. However, some points remain unresolved: it was not formally demonstrated that this effect is a direct consequence of E6 function, therefore cell lines stably expressing an inducible E6 oncogene should be produced to address this question. Moreover, it would also be interesting to establish whether this represents an exclusive function of the oncogenic

HPV types, or it is rather shared also by the benign viruses. The mechanism of cytoplasmic sequestration of p53 also needs to be determined. Since several lines of evidence point towards a role of the E6 protein in promoting the nuclear export of p53, experiments should be performed to address this possibility, for instance by analysing the effects of blocking nuclear export in E6 containing cells, or mutating the nuclear export signals on E6, E6AP and p53 proteins, or interfering with p53 ubiquitination mediated by the E6/E6AP complex.

Several PDZ-containing proteins have recently been shown to be crucial targets for the tumour-promoting activities of high-risk E6, one of them being the multi-PDZ protein MUPP1. We have demonstrated that high-risk HPV E6 targets MUPP1 for ubiquitin-mediated degradation and have also defined the sequence requirements for this activity on the E6 protein. We were able to identify the domains of HPV-18 E6 which mediate degradation of MUPP1, and to conclude that the E6AP ubiquitin ligase does not appear to be involved in this process. Interestingly, these results also confirmed that the low-risk HPV E6 proteins are able to target cellular substrates for ubiquitin-mediated degradation. Therefore, identification of the ubiquitin ligase(s) responsible for E6-mediated degradation of PDZ-containing proteins such as MUPP1 and Dlg is likely to provide important information on this activity of the low-risk viral types.

In the final part of this thesis, studies are described aimed at the elucidation of the complex and dynamic patterns of hDlg protein regulation in epithelial cells. The data presented 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 might also affect additional functions of hDlg that regulate cell proliferation. However, additional experimental work will be needed 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 transformed and invasive phenotype. Important questions still remain. First, the role of hDlg phosphorylation in regulating its degradation. Using different approaches, we have demonstrated that the endogenous hDlg protein needs to be phosphorylated in order to be degraded, and have also shown that β-TrCP binds to phosphorylated Dlg protein *in vitro* and *in vivo*. Since it is well known that β-TrCP selectively binds to its recognition sites when these are phosphorylated, it seems likely that this will also be the case for hDlg. Future work will include a fine mutational analysis to determine the specific residues on hDlg which determine its degradation, as well as the identification of the cellular kinase(s) responsible for regulating hDlg turnover. An appealing hypothesis would be that the GSK3β kinase, which binds APC and β-catenin and phosphorylates both, could induce degradation of hDlg as it does for β-catenin. However, preliminary results would indicate that this hypothesis is not correct, and an alternative kinase seems more likely to be involved.

Since we have started to dissect the cellular pathways that lead to hDlg ubiquitination, the next important question is whether high-risk HPV E6 exploits this same pathway to target hDlg for degradation, or a different ligase other than β-TrCP partners E6, similarly to what occurs in the degradation of p53. Recent data indicated that the SH3 domain of Dlg is dispensable for E6-mediated degradation, while the PDZ2 domain, which contains the E6-binding site, together with N-terminal sequences would rather appear to be involved in this process (Gardiol et al., 2002). Two different hypotheses can be formulated to explain these results. The simplest explanation would be that a yet unknown ubiquitin ligase binds hDlg within its PDZ2 domain and in turn is used by E6 to degrade hDlg. An alternative explanation is that E6 could either bridge the interaction between hDlg and β-TrCP, or alter its binding specificity thereby forcing the F-box protein to bind a hDlg domain, which it would not normally recognise. This strategy could enable E6 to bypass the normal cellular regulation of hDlg degradation, since it is likely that the E6-mediated association between β-TrCP and hDlg would not depend upon hDlg phosphorylation. Preliminary results show that E6 has no effect on the binding affinity of β-TrCP for the full-length Dlg protein *in vitro*, although it is possible that E6 promotes the interaction

of the ligase with a region of Dlg other than its normal consensus site, and this could be tested using Dlg mutant poteins. High-risk HPV E6 has previously been shown to redirect the specificity of the E6AP ubiquitin ligase towards p53, which is not a normal substrate of the HECT domain ligase. Interestingly, a similar case has also been reported where a viral protein, HIV Vpu, is able to bind β-TrCP and direct it towards the host protein CD4, which otherwise would not be bound by β-TrCP. The CD4 protein is thus targeted for ubiquitin-mediated degradation while Vpu is not degraded. (Margottin et al., 1998, and references therein). Therefore future work will be directed towards defining whether β-TrCP is also involved in the E6-mediated degradation of hDlg. Nonetheless, the work presented here provides a clear indication of the consequences of impairment of hDlg regulation in epithelial cells. Loss of hDlg regulation parallels the acquisition of a highly malignant phenotype, confirming the central importance of E6 targeting this cellular protein in the malignant progression of HPV-induced cervical dysplasia. We have discussed how most attempts at a therapeutic approach against cervical cancer have been historically directed towards inhibiting the activities of E6 towards p53. However, here we have demonstrated why these strategies would not necessarily be easily applicable. In the light of our findings, the design of alternative therapies for avoiding the deleterious effects of HPV oncoproteins upon their target cells should rather be aimed at blocking the interactions of the high-risk HPV E6 oncoproteins with PDZ-containing cellular targets.

Most of the work described in Part 1 and Part 3 is contained in the following articles:

Mantovani F. and Banks L. (1999) Inhibition of E6-induced degradation of p53 is not sufficient for p53 stabilisation in cervical tumour derived cell lines. Oncogene 18: 3309-3315.

Mantovani F., Massimi P., and Banks L. (2001) Proteasome-mediated regulation of the hDlg tumour suppressor protein in epithelial cells. J Cell Sci 114: 4825-4292.

MATERIALS AND METHODS

Plasmids.

pSL301-MUPP1 expression plasmid for *in vitro* transcription-translation has been described previously, as has the GW1-HAMUPP1 plasmid for expression of HA-tagged full-length MUPP1 protein *in vivo* (Lee et al., 2000).

GW1-9ORF1 plasmid has been described previously, as have the GW1-HA18E6, GW1-HA18E6V158A, GW1-HA18E6T/D-V/A and GW1-HA11E6, as well as the cloning of HPV-11 E6, HPV-18 E6 and of HPV-18 E6 mutant V158A in pGEX 2T for the expression of glutathione-S-transferase (GST) fusion protein in bacteria (Lee et al., 2000).

The cloning of HPV-18 E6 and p53 into pSP64 for *in vitro* translation has been described previously (Thomas et al., 1995; Pim et al., 1994).

The HPV-18 E6 mutants used in this study were ΔNT (Δ4-7), M2 (R10S, P11G), ΔD (Δ21-25), ΔA (Δ47-49), ΔE (Δ101-104), ΔF (Δ113-117), ΔG (Δ126-130) and ΔH (Δ144-149) and have been described previously (Pim et al., 1994). The wild-type and mutant E6s were cloned into pCDNA3 for expression *in vivo* (Pim et al., 1994; Gardiol and Banks, 1998). The two chimaeric constructs HPV-16.6 E6 and HPV-6.16 E6 have been described previously (Crook et al., 1991) and were also cloned into pCDNA3 for expression *in vivo* (Pim et al., 2000). The chimaeric construct HPV-6.18s E6 has also been described previously (Pim et al., 2000).

Dlg expression plasmids for the expression of GST-Dlg fusion proteins in bacteria were all obtained by cloning the entire Dlg ORF, or its portions, in frame with the glutathione S-transferase (GST) gene of pGEX-2TK expression vector at the *Bam*HI and *Eco*RI sites. To obtain Dlg deletion derivatives, defined portions of the Dlg/SAP97 ORF were PCR-amplified with specific primers using as template the Dlg/SAP97 cDNA clone described by Müller et al., 1995, according to the sequence therein reported. In particular, the full-lenght Dlg (GST-Dlg) and its deletion mutant derivative GST-CTDlg (which contains the Dlg residues downstream of aa 539), plus the HA-tagged CT-Dlg expression vectors were kindly provided by R. Javier and have been described previously (Lee et al., 1997; Gardiol et al., 1999). GST-DlgNT (containing amino acid residues 1-382) and GST-ΔSH3 (bearing a deletion encompassing residues 549-617) were kindly provided by D. Gardiol. HA-tagged Dlg expression plasmids FL-Dlg, NT-Dlg and ΔSH3-Dlg were kindly provided by D. Gardiol and have been described previously (Gardiol et al., 2002). They contain portions of the Dlg ORF cloned in-frame into an influenza virus hemagglutinin (HA) epitope-tagged pCDNA3 expression plasmid, and all have the HA tag on their amino-termini.

Myc-epitope-tagged β-TrCP and ΔFβ-TrCP expression plasmids were kindly provided by R. Benarous and have been described previously (Margottin et al., 1998; Hart et al., 1999). His-tagged ubiquitin expression plasmid was kindly provided by Christian Kühne.

Antibodies.

P53. Endogenous p53 protein was detected using a pool of the anti-p53 monoclonal antibodies pAb 1801, 1802 and 1803 (Banks et al., 1986). For immunoprecipitations, the C4 antibody was used, which is a polyclonal rabbit antiserum raised against the carboxyl terminal 14 amino acids of p53.

Dlg. Polyclonal antibodies against Dlg were kindly provided by P. Massimi, and were raised against a GST-Dlg N-terminus fusion protein, which comprises amino acid residues 1-222 of rat Dlg (Lee et al., 1997). For the first immunisation 200 μg of purified protein was injected into two New Zealand rabbits in incomplete Freund's adjuvant. In the following 6 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 (kindly provided by David Bredt) which is the closest PDZ domain containing family member to hDlg, thereby confirming the specificity of the antibody. The 6th boost of a single rabbit was used for all Western blot and immunoprecipitation experiments. For immunofluorescence analysis, cells were stained with 5 μg/ml anti-hDlg monoclonal antibody (2D11 Santa Cruz Biotech.).

MUPP1. Anti-MUPP1 rabbit polyclonal antiserum was generously provided by R. Javier and it has been described previously (Lee S. et al., 2000). This antibody was used used at 1:100 dilution for immunoprecipitation of *in vitro* translated MUPP1 protein.

Commercial antibodies. Anti-HA monoclonal antibody (Roche); rabbit polyclonal anti-HA antibody (Santa Cruz Biotech.); anti-Myc monoclonal antibody (9E11, Santa Cruz Biotech.); anti-hDlg monoclonal antibody (2D11 Santa Cruz Biotech.); anti-β galactosidase monoclonal antibody (Promega); goat polyclonal anti-βTrCP antibodies (Santa Cruz Biotech.). Biotinylated anti-mouse and anti-rabbit antibodies plus HRP-conjugated anti-goat antibodies and HRP-avidin were from DAKO. FITCH-conjugated goat anti-mouse secondary antibodies were from Molecular Probes.

***In vitro* transcription-translation and degradation assays.**

pSL301-MUPP1, pSP64-18E6, pSP64-p53 and pCDNA3-HA-FLDlg were transcribed and translated *in vitro* using the TNT-coupled rabbit reticulocyte system (Promega) and 10 μCi of [^{35}S]-cysteine (1000 Ci/mmol) (Amersham), as specified by the manufacturer. *In vitro* degradation assays were performed by mixing the specified *in vitro* translation reaction mixtures and incubating them at 30°C for the indicated times. Proteins were then immunoprecipitated with appropriate antibodies. After extensive washing with RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0.1% SDS, 2 μg/ml aprotinin, 100 μM TPCK and 50 μM TLCK) beads were eluted with Laemmli sample buffer, separated on SDS-PAGE gels, fixed in 10% acetic acid-30% methanol, dried and exposed to Kodak X-Omat film.

Production of GST-fusion proteins in bacteria and GST-pull down assays.

For protein production and purification, 50 ml of an overnight culture of *E. coli* strain BL-21 previously transformed with the appropriate expression plasmids were inoculated in a one to ten volume of Luria Broth containing ampicillin and grown at 37°C up to an OD of 0.6. Recombinant protein expression was induced for three hours with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (20 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% NP40, 2 μg/ml aprotinin, 100 μM TPCK and 50 μM TLCK) and the lysates were then cleared from cell debris by centrifugation. The GST-fusion proteins were then incubated for one hour with glutathione-conjugated agarose beads (Sigma) and bound proteins were then washed extensively with lysis buffer. The levels and purity of proteins were determined by SDS-PAGE and Coomassie Brilliant Blue R staining.

For MUPP1 pull down assays, equal amounts of different GST-E6 proteins bound to glutathione-linked agarose beads were incubated with COS7 cell lysates in RIPA buffer at 4°C for 2-4 hours, washed extensively with RIPA buffer, boiled in Laemmli sample buffer and separated by SDS-PAGE. Bound HA-MUPP1 protein was then determined by Western blot.

For β-TrCP pull down assays, equal amounts of different GST-Dlg proteins bound to glutathione-linked agarose beads were incubated with a fixed amount of the *in vitro* translated β-TrCP protein for one hour at room temperature in a final volume of 100 μl, in a binding buffer containing 50 mM Tris HCl pH 7.5, 100 mM NaCl and 2.5 mM EDTA. Bound proteins were washed extensively in PBS containing 1% NP40 before analysis by SDS-PAGE and autoradiography. Alternatively, GST-Dlg beads were first incubated for

15 minutes at 30°C with 10 µg of HaCaT cell extract in the presence of 200 µM ATP in a kinase buffer containing 20 mM Tris pH 7.5 and 10 mM $MgCl_2$ and 30 mM of the phosphatase inhibitor 4-nitrophenyl phosphate, washed twice in binding buffer supplemented with 30 mM of the phosphatase inhibitor 4-nitrophenyl phosphate and then used for binding assay as above. For the phosphatase assays, after incubation with the HaCaT cell extract, GST-Dlg beads were rinsed in PBS and incubated with 400 units of λ protein phosphatase (New England Biolabs) for 15 min at 30°C prior to binding assay. Quantitation of the binding levels was done by scanning the gels using a Packard Instant PhosphoImager.

***In vitro* phosphorylation of GST-Dlg fusion proteins.**

GST-Dlg fusion proteins were rinsed twice in kinase buffer and then incubated for 15 minutes at 30°C with 10 µg of HaCaT cell extract in the presence of 200 µM cold ATP and of 2.5 µCi of [γ-^{32}P]-ATP in kinase buffer. After extensive washing in kinase buffer the labeled proteins were analysed by SDS-PAGE and autoradiography.

Cell culture, genotoxic treatment and proteasome inhibition.

Human cervical carcinoma cell lines (HPV-16 positive CaSKi and SiHa, HPV-18 positive HeLa, SW756 and C4-I, HPV-68 positive Me-180 and HPV negative C33I), immortalised human skin keratinocytes HaCaT, human mammary adenocarcinoma MCF7 cells, human fibrosarcoma HT1080 cells, human embryonic kidney 293 cells and monkey kidney COS7 cells were all maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 10% CO_2.

Primary baby rat kidney (BRK) cells were obtained from 9 day-old Wistar rats, and subsequently transformed with HPV-16 E7 plus EJ-ras as described previously (Massimi et al., 1997). BRK cells were grown under the same conditions as the epithelial tumour-derived cell lines.

Establishment of HPV-16 immortalised cell lines W12, V1/G and AC89/E2 has been described previously (Stanley et al., 1989; Bouvard et al., 1996; Storey et al., 1992). Keratinocyte cell lines were grown in a 3:1 mixture of DMEM and Ham F12 medium, supplemented with 10% fetal calf serum, 10 ng/ml epidermal growth factor, 10^{-10} M cholera toxin, 0.4 µg/ml hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 10% CO_2.

Rat prostate cancer cell lines G, AT1 and MatLyLu were grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 nM dexamethasone (Sigma) at 37°C and 10% CO_2.
For genotoxic treatment, cells were incubated with 10 µg/ml mitomycin C (Sigma) for 18 hours. For proteasome inhibition, growing cells were treated with either CBZ (N-CBZ-leu-leu-leu-al, Sigma) or LL (N-acetyl-leu-leu-norleucinal, Sigma) at a final concentration of 50 µM, or with 25 µM lactacystin (Calbiochem) for 2 or 4 hours prior to harvesting for subsequent analysis.

Western blotting.
Cells were rinsed in ice-cold PBS, lysed on ice in buffer A (50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% NP40, 2 µg/ml aprotinin, 100 µM TPCK and 50 µM TLCK) for 10 min and then cleared by centrifugation at 13.000 rpm for 10 min at 4°C. Protein concentrations were determined using the Bio-rad Protein Assay System, and equal amounts were then separated on SDS-PAGE and transferred to nitrocellulose membrane. Proteins were detected with the appropriate specific antibodies, and developed with the Amersham ECL System according to the manufacturer's instructions.

Immunoprecipitations.
Cells were rinsed in ice-cold PBS and lysed on ice in buffer B (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0,5% NP40, 5% glycerol, 100 µM TPCK, 50 µM TLCK and 30 mM 4-nitrophenyl phosphate). Cell lysates were cleared by centrifugation at 13.000 rpm for 10 min, incubated with appropriate specific antibodies for times ranging from 1 hr to overnight at +4°C, and subsequently mixed with either protein A- or protein G-agarose beads (Amersham Pharmacia biotech.) for 40 min. Where necessary, the anti-Dlg and anti-βTrCP antibodies were covalently bound to the protein A or protein G using 5 mg/ml dimethylpimelimidate (Pierce) as crosslinker.

Transfection of mammalian cells.
293 cells were transiently transfected by standard calcium phosphate precipitation procedure. COS7 cells were transfected with Lipofectin or Lipofectamine (Gibco BRL) as recommended by the manufacturer.

***In vivo* degradation assays.**

MUPP1.

COS7 cells were lipofected with 1 μg of GW1-HAMUPP1 expression vector and 4 mg of either GW1 empty plasmid or a GW1 plasmid expressing HA epitope tagged wild-type HPV-11 E6, wild-type HPV-18 E6 or mutant HPV-18 E6 proteins. 293 cells were transfected with 2 μg of HA-MUPP1 expression vector and with either 2 or 4 μg of the appropriate pCDNA3-HPV E6 expression vectors. 250 ng of CMVLTR-LacZ expression vector were also included to allow normalisation of different experiments for transfection efficiency. 36 hours post-transfection, the cells were either treated for two hours with the proteasome inhibitors LL and CBZ or with DMSO as a control, and subsequently harvested and analysed by Western blotting with anti-β galactosidase and anti-HA antibodies as above described.

Dlg. 293 cells were transfected with 2 μg of the appropriate HA-Dlg expression vector and with either 2 or 4 μg of Myc-tagged β-TrCP expression vectors, together with 250 ng of CMVLTR-LacZ expression vector to allow normalisation for transfection efficiency. Cells were then treated and analysed as above described.

^{35}S metabolic labeling of cellular proteins and pulse-chase experiments.

At 24 hours post transfection, cells were incubated for 1 hour in culture medium lacking methionine and cysteine, and then 100 μCi per ml of [^{35}S]-methionine/cysteine (Tran^{35}Slabel, ICN) was added to the same medium for 1 hour. Cells were either washed in ice-cold PBS and harvested (time zero) or incubated in complete medium for the times indicated and subsequently harvested and lysed in RIPA buffer. Cell lysates were immunoprecipitated with rabbit polyclonal anti-HA antibody pre-bound to protein A-agarose beads. After extensive washing with RIPA buffer, beads were eluted with Laemmli sample buffer, separated on SDS-10% PAGE gels, fixed in 10% acetic acid-30% methanol, dried and exposed to Kodak X-Omat film. Quantitation was performed using a Packard Instant PhosphoImager.

Phosphatase assays.

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

Immunofluorescence assays.

p53: cells were fixed for 20 min with methanol at -20°C, rehydrated in PBS, then reacted for 1 hour with the same monoclonal antibody pool used for Western blotting. After incubation with biotinylated anti-mouse antibody diluted at 1:200, and subsequently with streptavidin-FITCH diluted at 1:50 (Southern Biotechnology Associates) the cells were mounted using mounting medium for fluorescent microscopy. Pictures were taken using a Zeiss Axiovert 35 microscope and a Contax camera.

hDlg: cells were washed in PBS, fixed in 3% paraformaldehyde in PBS at RT for 20 min, permeabilised for 5 min in 0.1% Triton-PBS, stained with anti-hDlg monoclonal antibody (5 µg/ml) for 1 hour at 37°C 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. $2.5x10^4$ cells/ml were suspended in growth medium containing either 0.5% or 1% (w/v) noble agar in 60 mm diameter Petri dishes. Colonies were counted after 10 days.

RNA extraction, RT-PCR and cycle sequencing of p53.

Total cellular RNA was isolated from cultured cells with RNAzolB (BioGenesis) according to the manufacturer's instructions, DNAse treated and quantified. 5 µg of total RNA were reverse transcribed for 1h at 39°C using 200U of Moloney murine Leukaemia virus reverse transcriptase (Gibco BRL) and the p53 specific antisense primer 5'GGCAGGTCTTGGCCAGTT3'. 30 cycles of amplification were then performed using the oligonucleotides:

upstream: 5'CTCTGAGTCAGGAAACATT3'

downstream: 5'AAGGGACAGAAGATGACAG3'.

Amplification conditions were: 1 min at 95°C, 1 min at 58°C and 1 min at 72°C. After purifcation, the RT-PCR products (259 bp) were then subjected to cycle sequencing with the internal oligonucleotide primer 5'GATATTGAACAATGGTTC3', using the Amersham Thermosequenase kit and [^{33}P]-labelled ddNTP terminators; cycling conditions were: 1 min at 95°C, 1 min at 55°C and 2 min at 72°C for 45 cycles.

hDlg RT-PCR and Southern Blot analysis.

Total RNA was extracted as described above and then reverse-transcribed using specific antisense primers:

hDlg antisense primer: 5'GTAGAGCTTGGAAGGCTGGAA3'

TBP antisense primer: 5'GGTACATGAATTCCATTACGTCGT3'

13 cycles of amplification were then performed using either **hDlg primers**:

upstream: 5'ATGCCGGTCCGGAAGCAAGAT3'

downstream: 5'GTAGAGCTTGGAAGGCTGGAA3',

or **TBP primers** (Massimi et al., 1997):

upstream: 5'GCTGCGGGATCCATGAGGATAAGA3'

downstream: 5'GGTACATGAATTCCATTACGTCGT3'.

Amplification conditions were: 1 min at 95°C, 1 min at 56°C and 1 min 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 labeled internal oligonucleotides:

hDlg probe: 5'CAGACGGCTTTGAACGATGTA3'

TBP probe: 5'TGGCTCAGAATTCCTAAATTGTT3'.

Quantitation of the mRNA expression was done by scanning the Southern blots using a Packard Instant PhosphoImager.

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