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DOCTEUR DE L'UNIVERSITE LOUIS PASTEUR DE STRASBOURG I
par

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Cancer du col de l'utérus: Adressage tumoral et définition de ligands peptidiques de l'oncoprotéine E6 de HPV16.

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ABBREVIATIONS

BSA	Bovine serum albumin
cDNA	complementary DNA
CDK	Cyclin-dependant kinase
CDR	Complementarity determining regions
cfu	Colony forming units
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
DT	Diphtheria Toxin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
Fab	Antigen-binding fragment
FACS	Flow assisted cell sorting
FCS	Fœtal calf serum
FIGO	Féderation Internationale de Gynécologie et d'Obstetrique
GST	Glutathione-S-transferase
HPV	Human papilloma virus
IPTG	Isopropyl-β-D-thiogalactopyranoside
IgG	Immunoglobin, class G
KDa	Kilo Dalton
Kbp	Kilo base pair
M13	M13 filamentous phage
mAb	monoclonal antibody
MBP	Maltose-binding protein
min	Minutes
mRNA	Messenger RNA
NP-40	Nonidet P40
ORF	open reading frame
pIII / pVIII	M13 phage minor coat protein III and major coat protein VIII
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction

PEI	Polyethylenimine
PVC	Polyvinyl chloride
scFv	Single-chain variable region fragment
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamid gel electrophoresis
ssDNA	single stranded DNA
TBS	Tris-buffered saline
TMB	3-3,5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
tu	Transducing units
UV	Ultra-violet

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophane
Y	Tyr	Tyrosine

Cell lines

SiHa cells Derived from human squamous cell cervical carcinoma
Contain an integrated HPV 16 genome

HeLa cells Derived from human cervical epithelial cell adenocarcinoma
Contain HPV-18 sequences

CaSki cells Derived from a metastatic site (small intestine) of an epidermoid carcinoma
Contain an integrated HPV 16 genome

Saos-2 cells Derived from an osteosarcoma
p53 null

H1299 cells Derived from a metastatic site (lymph node) of a non-small cell lung carcinoma
p53 null

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RESUME EN FRANCAIS

Dans le monde, le cancer du col de l'utérus peut compter jusqu'à 25% des cancers chez la femme et représente le deuxième cancer mortel (Burd, 2003; NCI, 2003a; NCI, 2003b). Les études épidemiologiques ont confirmé que le facteur le plus associé au développement de ce cancer est l'infection par une sous-classe de virus du papillome humain (HPVs) dits de "haut-risque", notamment les types 16 et 18. Cette association est très forte car 99,7% des cas du cancer du col de l'utérus squameux sont liés à l'infection par HPV (Walboomers et al., 1999). La méthode la plus répandue pour le dépistage du cancer du col de l'utérus est le test Pap-smear (Papanicolaou-stained smear). Cependant la fiabilité du dépistage dépend largement de la qualité de foration du personnel impliqué dans la collecte d'échantillons et l'interprétation des données (Jordan and Monaghan, 2004; Sherman et al., 1994). Des améliorations techniques ont augmenté la fiabilité des résultats et des systèmes automatiques sont en cours de développement.

Le cancer est un terme générique pour définir des néoplasmes malins qui apparaissent, en partie, lors du dérèglement du cycle cellulaire et de la suppression de la mort cellulaire programmée, l'apoptose. Le cycle cellulaire est divisé en 4 phases. Les phases de synthèse (S) et mitose (M) sont espacées de la phase G_1 et G_2. La transition entre ces phases est soumise à des points de contrôle qui sont médiées par les cyclines et les kinases dépendantes de cyclines (CDKs) (Ivanchuk and Rutka, 2004). L'activité des CDKs est dictée par les cyclines qui sont produites et dégradées par le protéasome 26S en fonction de signaux exta- et intracellulaires. Deux protéines exercent un contrôle important sur le cycle cellulaire : la protéine Rb et p53 (DeWolf and Gaston, 2004). En présence de signaux cellulaires spécifiques, ces protéines exercent un contrôle négatif sur le cycle cellulaire. p53 a un effet positif sur la réparation des dommages de l'ADN et l'apoptose (Harms et al., 2004). Ces éléments ont une demi-vie cellulaire très réduite grâce à leur dégradation par le protéasome 26S. Les protéines désignées pour ce type de dégradation sont modifiées par addition séquentielle de monomère d'ubiquitine (Bajorek and Glickman, 2004). Les chaînes de poly-ubiquitine sont reconnues par une sous-unité du 26S proteasome qui engendre ensuite la dégradation du substrat. La poly-ubiquitination nécessite une ubiquitine-ligase spécifique et pour le cas de p53, par exemple, il a été démontré que Mouse Double Minute 2 (MDM2) est l'ubiquitine-ligase responsable pour l'ubiquitination de p53 dans des cellules normales (Moll and Petrenko, 2003).

Suite à l'infection par HPV des cellules des couches basales de l'épiderme, l'ADN viral s'établit comme un élément extrachromosomal représentant la phase latente d'infection (Burd, 2003). Pendant la phase de latence, seuls les gènes précoces E1, E2, E5, E6 et E7 sont exprimés. Le produit du gène E2, la protéine E2, inhibe la transcription des gènes depuis le promoteur majeur précoce formant une boucle auto-regulatrice. Au moment de la différentiation cellulaire des keratinocytes, des facteurs encore non-définis induisent l'expression de gènes depuis le promoteur majeur tardif qui n'est pas réprimé par la protéine E2. Ce changement marque le début de la phase productive du cycle viral, période au cours de la quelle les gènes qui codent pour les protéines structurales (L1 et L2) de la capside virale sont exprimés et l'ADN génomique viral est amplifié (Longworth and Laimins, 2004b).

Comme nous l'avons déjà souligné, l'immortalisation cellulaire n'est provoquée que par les HPV de type haut-risque et seulement à la suite de l'intégration du génome viral dans le génôme de la cellule hôte. L'intégration perturbe ou délète le gène E2, supprimant ainsi son expression et son contrôle négatif sur le promoteur majeur précoce. Ceci induit l'expression non-contrôlée des gènes E6 et E7 qui sont responsables de l'immortalisation de la cellule hôte (Munger et al., 2004). La protéine E7 induit la dégradation de la protéine pRb, produit du gène de susceptibilité au rétinoblastome. Cette protéine contrôle le point de restriction du cycle cellulaire pendant la phase G_1. La dégradation de pRb s'effectue par le protéasome 26S, par un mécanisme encore mal compris (Longworth and Laimins, 2004b). Ainsi, E7 a un effet positif sur la transition entre les phases G_1 et S, et les phases G_2 et M. E7 agit également sur les désacétylases de histones, une activité liée au maintien de l'ADN viral en élément extrachromosomal (Longworth and Laimins, 2004b). La protéine E7 est dégradée elle-même par le protéasome 26S dans un système dépendant de l'ubiquitine. Cette dégradation est sous contrôle du complexe ubiquitine-ligase Skp1-Cul-F-box et de l'ubiquitine-ligase SOC1 ("Suppressor Of Cytokine Signaling-1") (Kamio et al., 2004; Oh et al., 2004a).

La protéine E6 issue de HPV de haut-risque induit la dégradation de p53 par association à la protéine "E6 Associated Protein" (E6-AP). Cette dernière est une ubiquitine ligase qui ne cible pas p53 pour l'ubiquitination dans des cellules normales mais seulement en présence de E6 de haut-risque. E6 interagit aussi avec plusieurs autres protéines cellulaires et, pour certaines d'entre elles, il a été possible de définir le motif consensus nécessaire pour cette interaction. Dans un premier temps, les protéines à motif composé de leucines (charged

leucine motif) ont été identifiées et regroupent E6-AP, E6 Binding Protein (E6BP), MultiCopy Maintenance Protein 7 (MCM7), Paxillin and Tumour Necrosis Factor Receptor 1 (TNF R1) (Chen et al., 1998; Elston et al., 1998; Vande Pol et al., 1998). Un deuxième groupe qui interagit avec l'extrémité C-terminale de la protéine E6 réunit les protéines à domaine PDZ (**P**SD-96/**D**lg/**Z**O-1) et inclut également la famille de guanylate kinase associée aux membranes (MAGUK) (Watson et al., 2003). Plusieurs autres protéines interagissent également avec E6 sans que le motif d'interaction ne soit identifié. Dans ce groupe sont réunies les protéines p300/Creb-Binding Protein (CBP), TyK2, Interferon Regulatory Factor-3 (IRF-3), Bak et Myc (Horikawa and Barrett, 2003; Kuhne and Banks, 1998; Li et al., 1999; Ronco et al., 1998; Veldman et al., 2003; Zimmermann et al., 1999). La protéine E6 est elle même ubiquitinée et dégradée par le portéasome 26S.

Les protéines E6 et E7 peuvent induire l'immortalisation de cellules et les étapes clefs peuvent être résumées en trois points. D'abord E7 agit sur pRb ce qui a pour conquence, une division cellulaire sans contrôle puis, E6 en association avec E6-AP induit la dégradation de p53, inhibant ainsi les signaux pro-apoptotiques. E6, en association avec Myc, induit ensuite la transcription du gène de la sous-unité catalytique de la télomérase (hTERT) prolongeant ainsi indéfiniment le nombre de division cellulaire.

Actuellement, les traitement utilisés pour le cancer du col de l'utérus manque de spécificité qui induit des effets secondaires et réduit leur efficacité. De plus, l'interaction de E6 de haut-risque avec tous ses partenaires cellulaires n'est pas encore pleinement compris ce qui est un frein pour le développement de nouvelles molécules thérapeutiques. C'est dans ce contexte scientifique que nous avons utilisé la technologie du phage display pour la définition de peptides capable de cibler et d'internaliser des cellules issues du cancer du col de l'utérus puis la définition de ligands peptidiques de l'oncoprotéine E6 issue du HPV de type 16 (16E6).

La technologie du phage display décrite en premier par Parmley et Smith en 1988, se base sur la présentation d'un peptide ou d'une protéine sur la capside d'un bactériophage (Parmley and Smith, 1988). Cette configuration permet la liaison phénotype-génotype. En effet, la molécule présentée sur la capside est codée par un gène introduit dans l'ADN du phage lui même. Cette liaison entre le phénotype et le génotype permet une grande efficacité pour le criblage de banque car la sélection d'un clone permet, après séquençage génétique, la déduction du peptide présenté. Le bactériophage filamenteux M13 est le plus utilisé pour le criblage de banques de peptides ou de protéines. Dans ce cas, la protéine de la capside pIII ou pVIII de M13 est choisie pour la présentation. La première méthodologie utilisée pour le criblage d'une banque de phage display est basée sur la sélection par affinité. La banque de phages est mise au contact de la cible puis, les particules fixées sont isolées et amplifiées après infection bactérienne. En augmentant la stringence des cycles de sélection successifs, on enrichit la sous population de phages d'affinité supérieure pour la cible.

La liaison entre génotype et phénotype pour le criblage de banques est employée dans diverses autres technologies. Par exemple, le double-hybride et le ribosome- ou ARNm-protéine display (Fields and Song, 1989; Mattheakis et al., 1994; Nemoto et al., 1997; Roberts and Szostak, 1997). Les technologies basées sur le principe du double-hybride ont l'avantage

de réaliser le criblage dans un contexte intracellulaire. Cependant, cette approche ne peut être envisagée dans le cas de molécules extracellulaires et ne pemet pas de modifications de l'environment de sélection, l'augmentation de la stringence par exemple. Les approches par ribosome- ou ARNm-protéine display n'ont pas l'inconvénient de la restriction de diversité contrairement aux approches de phage-display et de double hybride. En effet, ces approches sont limitées par l'étape de transformation de la banque sous forme plasmidique de cellules bactériennes, cellules de levure ou cellules mammifères. Par contre, le ribosome- ou ARNm-protéine display nécessite un environnement dépourvu d'activité RNase. Par rapport à ces approches, le phage display à l'avantage d'être un système simple et robuste pour lequel les conditions de sélection peuvent être coisies et optimisées.

Le phage display a été employé pour la sélection de protéines et/ou de peptides. Cette technologie a notamment révolutionné le domaine des anticorps humains (Watkins and Ouwehand, 2000). Des banques de fragments d'anticorps humains ont ainsi pu être criblées, générant des molécules avec des applications thérapeutiques. Les banques phage-peptides ont été utilisées pour la définition d'épitopes d'anticorps (Choulier et al., 2001) et de ligands d'enzymes (Ruan et al., 1998). Des interactions entre protéines intracellulaires ainsi que des ligands de récepteurs extracellulaires ont pu êtres isolés (Pelletier and Sidhu, 2001). Ces peptides donnent à la fois des informations sur les contacts établis entre protéines et offrent la possibilité d'être utilisés comme molécules agonistes ou antagonistes de cette interaction. Des peptides et/ou des anticorps recombinants ont également été sélectionnés pour le ciblage tumoral (Nilsson et al., 2000). Le processus de transformation cellulaire induit des modifications cellulaires qui se traduisent par une altération des molécules présentées à la surface des cellules. Le criblage des banques de phages a permis la sélection de peptides et d'anticorps qui ciblent et s'internalisent dans des cellules tumorales.

En utilisant la technologie du phage display, nous avons entrepris, dans un premier temps, la définition de peptides d'adressage et d'internalisation dans les cellules issues du cancer du col de l'utérus (SiHa). Dans un deuxième temps, cette approche a été appliquée dans la définition de ligands peptidiques de l'oncoprotéine 16E6. Les peptides internalisants ont permis le reciblage de la toxine diphtérique dans les cellules SiHa. Les peptides ligands de la protéine 16E6 ont permis l'identification de domaines de p53 important pour sa dégradation.

L'internalisation cellulaire est un prérequis dans de nombreuses stratégies thérapeutiques modernes utilisant des drogues, peptides, polypeptides ou du matériel génétique. La délivrance spécifique aux cellules cibles est primordiale pour limiter les effets secondaires. La méthodologie que nous avons utilisée repose à la fois sur la banque utilisée et sur la stratégie employée pour le criblage et favorise l'obtention de particules qui sont internalisés par des cellules humaines (Robinson et al., 2004). La banque est composée de peptides contraints présentés de manière multivalente sur la surface des particules phagiques. Nous avons criblé cette banque sur la lignée cellulaire SiHa. Les peptides ainsi isolés, présentent une spécificité pour plusieurs lignées de cellules issues de cancers du col de l'utérus (SiHa, CaSki et HeLa). Nous avons pu établir que ces peptides retiennent leur capacité d'internalisation en fusion avec la protéine rapporteuse EGFP (Enhanced Green Fluorescent Protein). Cependant, toutes les molécules internalisées empruntent la voie d'endocytose qui les dirige vers les lysosomes où elles sont dégradées. Une thérapie se basant sur ces peptides doit obligatoirement prendre en considération ce phénommène. Ainsi, pour contourner ce problème nous avons exploré la possibilité d'internalisation de la toxine diphtérique. Cette protéine, issue de la bactérie *Corynebacterium diphtheriae*, est une toxine bactérienne de type AB (Menetrey et al., 2005). Ces toxines sont composées de deux domaines: le domaine A qui contient l'activité enzymatique responsable pour la toxicité et qui est transloqué dans le cytosol des cellules ciblées et le domaine B inséré dans la membrane endoplasmique. Le segment B est constitué en réalité de deux domaines distincts qui servent à la liaison au récepteur cellulaire et à la translocation membranaire. Cette configuration offre la possibilité de remplacement du domaine responsable de la liaison au récepteur cellulaire par une autre molécule, par exemple, des anticorps recombinants, des interleukines ou encore des peptides pour le ciblage tumoral. Ces molécules recombinantes ont permis le reciblage de la toxicité de la toxine diphtérique aux cellules cancéreuses. De plus, il a été démontré que des peptides fusionnés au domaine A de cette protéine sont transloqués dans le cytosol des cellules ciblées (Stenmark et al., 1991). Dans ce but, nous avons modifié la protéine diphtérique en remplaçant le domaine de liaison au récepteur cellulaire par un des peptides isolés par le phage-peptide display. Nous avons ensuite étudié la faisabilité d'utilisation du domaine de translocation membranaire de la toxine diphtérique pour délivrer les peptides dans le cytosol des cellules SiHa. Par microscopie immunofluorescence, nous avons démontré le ciblage et l'internalisation des cellules SiHa par cette molécule recombinante mais, la translocation de la molécule recombinante vers le cytosol n'a pas pu être visualisée. Une des explications possibles est qu'après endocytose, la majorité des molécules de la protéine

diphtérique est rapidement dirigée vers le lysosome puis dégradée et le faible nombres de molécules transloquées n'est pas suffisant pour produire un signal détectable par immunofluorescence. Nous envisageons d'étudier la validité de cette hypothèse en utilisant des molécules marquées à des isotopes radioactifs et ainsi définir la déstinée de ces molécules d'un manière plus quantitative.

Dans l'autre axe de recherche, nous avons utilisé le phage display pour définir des peptides ligands de l'oncoprotéine E6 HPV16. Cette protéine est connue pour être difficile à purifier. Le travail effectué par G. Travé et collaborateurs dans notre laboratoire a permis d'obtenir une protéine recombinante, pure et correctement repliée (Nomine et al., 2001b). Utilisant cette protéine, nous avons criblé une banque de phages-peptides linéaires. Parmi les séquences isolées, un groupe représente une forte homologie avec la région 261 à 265 du domaine central (core domaine) de p53. Nous pensons que cette région, qui comprend les acides aminés ^{261}SGNLLG^{265} de p53 constituerait le second domaine de liaison de E6 avec p53. En effet, il a été démontré que E6 seule lie la région 376-384 de p53 et en présence de E6-AP, E6 lie également une région située dans le domaine central de p53. Pour valider cette hypothèse, nous avons d'abord démontré la liaison entre 16E6 et le peptide 261-265 en utilisant l'approche d'immunoprécipitation et de la rétention spécifique sur gel. Les amino acids de p53 correspondant à cette région ont été ensuite mutés ponctuellement en alanine. Seul le mutant L265A a été protégé de la dégradation par 16E6 alors que p53 wt et les autres mutants ont été rapidement dégradés in vitro dans des extraits de réticulocyte de lapin. Le mutant L265A est également résistant à la dégradation dans les cellules H1299, lignée dépourvue de p53. Au contraire, dans les cellules Saos-2, lignée également dépourvue de p53, la protéine p53 L265A est instable en absence de 16E6. Nous suggérons que cette grande instabilité de p53 L265A dans les cellules Saos-2 est dû à un facteur, encore non-identifié, présent dans les cellules Saos-2 mais absent dans les réticulocytes de lapin et les cellules H1299.

La représentation tri-dimensionnelle de la structure du domaine central de p53 a permis d'observer que la région 261-265 est en contact avec la région 99-107. Des études réalisées par d'autres groupes de recherche ont montré que cette région de p53 (99-107) est impliquée dans la dégradation de p53 dépendante et indépendante de 16E6. La mutation de cette région de p53 empêche la dégradation par 16E6 et, de plus, contribue à la stabilité de p53, dans les cellules Saos-2.

L'ensemble de ces informations suggère fortement l'importance de ces deux régions dans la dégradation de p53. Le comportement inattendu de la protéine p53 L265A dans les cellules Saos-2 est extrêmement intéressant car les données obtenues indiquent que l'amino acide L265 est impliqué dans la dégradation de p53 dépendante de E6 et indépendante de E6. L'étude du facteur cellulaire putatif présent dans les cellules Saos-2 qui serait responsable du mutant L265A sera precieuse dans le décryptage du mécanisme de dégradation de p53. La

région 99-107 est également impliquée dans la dégradation de p53, mais représente un système commun des voies de dégradation dépendante et indépendante de E6.

Ces travaux présentés ici attestent de la richesse des résultats obtenus par la technologie du phage display. Les peptides ainsi obtenus ont servi à l'adressage spécifique de cellules tumorales et ont généré des données précieuses pour la compréhension de la dégradation de p53.

GENERAL INTRODUCTION

1. Cervical cancer

In developing countries cervical cancer may account for up to 25% of female cancers and world-wide it is the second most fatal cancer in women. In the United States of America (USA), cervical cancer is the third most common female cancer, and over 10,000 cases are expected to have occurred in 2004, and it is projected that these will lead to nearly 4000 fatalities (Burd, 2003; NCI, 2003b). On the other hand, in the UK, cervical cancer is only the eleventh most common cause of cancer in women, representing 2% of female cancers and 927 deaths in 2002 (Jordan and Monaghan, 2004) and in France represted 2.9% of female cancers and resulted in 1 000 deaths in the year 2000 (INVS, 2003). The difference in the number of fatal cases and relative importance of cervical cancer between countries is largely attributed to the implementation of comprehensive screening programs (Laara et al., 1987).

The etiological factor for the development of preinvasive and invasive cervical cancer is infection with a tumourigenic sub-family of human papillomavirus (HPV) (Jordan and Monaghan, 2004). Although it was postulated in the mid 1970's that there was a link between HPV and cervical cancer, it was not until 1983 and 1984 that zur Hausen and co-workers isolated the first HPV types (16 and 18 respectively) directly from cervical cancer biopsies (Boshart et al., 1984; Durst et al., 1983). This association between HPV and cervical cancer was jointly recognised in 1996 by the World Health Organisation, the European Research Organisation on Genital Infection and Neoplasia and the National Institutes of Health Consensus Conference on Cervical Cancer. Additionally, more sensitive methods of detection have associated HPVs with 99.7% of cervical squamous cell cancer world-wide (Walboomers et al., 1999).

Type of genital lesion	Type of HPV
Condylomata acuminata (genital warts)	**6**, **11**, 42, 44, 51, 53, 83
Intraepithelial neoplasias	6, 11, **16**,18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 73, 74, 79, 81, 82, 83, 84
Cervical and other anogenital cancers	(6, 11), **16**, **18**, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68, 69

Table 1.
Genital lesions and HPV types.
Most prevalent HPV types associated with warts, intraepithelial neoplasias and cancers are in bold, high-risk HPV types are underlined. HPV types 6 and 11 association with anogenital cancers is rare which is indicated by the brackets.
Adapted from zur Hausen 2002 and Burd 2003.

FIGO stage	Comment
0	Preinvasive carcinoma *in situ*
I	Confined cervical carcinoma
IA	Invasive carcinoma (diagnosed only by microscopy)
IA1	Stromal invasion < 3mm deep and 7mm wide
IA2	Stromal invasion > 3mm deep and 7mm wide
IB	Clinically visible lesion
IB1	Clinically visible lesion < 4 cm
IB2	Clinically visible lesion > 4 cm
II	Tumour invasion beyond uterus but not pelvic wall or lower vagina
IIA	- without parametrial invasion
IIB	- with parametrial invasion
III	Tumour extends to pelvic wall or lower vagina
IIIA	Tumour extends to lower vagina but not pelvic wall
IIIB	Tumour extends to pelvic wall and causes nephrosis or non-funct. kidney
IV IVA	Tumour invades mucosa of bladder / rectum / beyond pelvis
IVB	Distant metastasis

Table 2.
Classification of cervical neoplasia.
Clinical parameters are used to classify cervical neoplasia following the Féderation International de Gynécologie et d'Obstetrie (FIGO) system. Other systems do exist and include the Tumour-node-metastasis (TMN).
Adapted from Jordan and Monaghan 2004.

HPVs are transmitted primarily by contact and, in the anogenital tract, by sexual contact. Early sexual activity and multiple sexual partners increase the risk of HPV infection. Furthermore, condom usage is a relatively ineffective method in protecting against HPV infection as these may be transmitted by contact with unprotected areas such as the labia, scrotum or anus (Burd, 2003). Additional factors increase the risk of developing invasive cervical cancer, the most important being smoking. Indeed, the mutagenic components of cigarette smoke and the local immune suppression induced by smoking may contribute to persistence of HPV infection and cellular transformation (Yang et al., 1996).

HPVs are double stranded DNA viruses and, on the basis of DNA sequence, more than 200 types have been distinguished. Furthermore, these 200 types of HPV can be grouped into cutaneous or mucosal types according to the tissues they target and low- or high-risk types according to their tumourigenic potential (zur Hausen, 1999). Cutaneous types target the skin of the hands and feet whereas the mucosal types infect the anogenital tract or the lining of the mouth, throat and respiratory tract. Low-risk types produce benign localised warts, whereas high-risk types undergo malignant progression. For example, HPV5 and 8 are high-risk cutaneous types as these are associated with the developement of epidermodysplasia verruciformis, a rare, yet historically important, epidermological condition. Patients suffering from this condition develop lesions similar to warts and are prone to develop non-melanoma skin cancer. This condition provided the earliest indications that HPVs contributed to tumourigenesis (Jablonska et al., 1966; Pass et al., 1977; Pfister et al., 1981). In the anogenital tract, low-risk HPVs (including types 6 and 11) have been found in genital warts (condyloma accuminata) and non-malignant lesions whereas high-risk HPVs (including types 16 and 18) have been associated with squamous intraepithelial lesions (SIL) which are usually cleared within 12 months by the immune system (Table 1). Persistent SILs may progress to high-grade SIL and then to carcinoma *in situ* (zur Hausen, 2002). Subsequent carcinoma progression dealing specifically with cervical carcinoma is detailed in Table 2 following the "Féderation Internationale de Gynécologie et d'Obstetrique" (FIGO) staging system. Thirty to seventy percent of the precursor lesions (carcinoma *in situ* or cervical intraepithelial neoplasia) will progress into invasive cancer over a period of approximately 10 years.

The methods employed for cervical cancer screening are commonly based on cytological and colposcopic analyses. Historically, the most important of these tests has been the Papanicolaou-stained (Pap) smear introduced in 1949 (Papanicolaou, 1949). This

technique is still employed today (Burd, 2003; Jordan and Monaghan, 2004) however, its major limiting factor is the human element involved in sample gathering (Jordan and Monaghan, 2004) and interpretation of the results (Sherman et al., 1994). Modifications have been made to increase the fidelity of the results obtained, for example by using monolayer cytology (Hutchinson et al., 1994). Additionally, the technical definitions vary from country to country and so in recent years there has been a trend for global harmonisation of the terminology employed to describe results (Burd, 2003; Jordan and Monaghan, 2004). In countries that lack experienced personnel, however, the risk of human error is increased (zur Hausen, 2002). Such countries should benefit from an automated screening system, yet the additional cost of these may prove prohibitive. Furthermore, analysis of screening records in Bristol (England) by Raffle *et al*. projects that 1000 women are required to be screened for 35 years to prevent 1 death. For this prevented death, 150 women had an abnormal result, over 80 where referred and of these 50 were treated (Raffle et al., 2003). This points to the number of false positives generated by this technique but also to the detection and treatment of SILs which would have been reabsorbed without external intervention as discussed previously. The impact of these unnecessary interventions on the health of the women concerned is still not known.

Recently HPV DNA and/or RNA have been used to detect HPV in biopsy tissues. Several methods have been employed and include type-specific or non-specific PCR using oligonucleotide primers and various hybridisation techniques. In general, detection of HPV DNA or RNA has been demonstrated to be methods acting in complement to the Pap-smear technique. Indeed, high-risk HPV testing has been found to be more sensitive than cytomorphological tests and produces accurate negative results for stage 0 carcinoma *in situ* and above (Table 2) allowing less frequent testing in women who present both cytologically normal and undetectable levels of HPV (Bory et al., 2002; Zielinski et al., 2003). The HPV testing also allows detection of viral load, an indication of prepondancy for the development of cervical intrepithelial neoplasia (Snijders et al., 1998). However, it is possible that using these tests for primary screening increases the number of positive results for patients who have transient, asymptotic and non-consequential HPV infection not identified by cytological testing (NCI, 2003a).

Treatment and prognosis of cervical cancer is determined by histological stage, tumour volume and spread (NCI, 2003b). Non-invasive intraepithelial lesions (Stage 0, Table 2) are treated by laser or cryotherapy and non-invasive squamous lesions using loop electrosurgical excision procedure. Small invasive lesions (Stage IA, table 2) are treated by excisional cone biopsy and radical hysterectomy. Invasive carcinomas (Stage IB and II, Table 2) are treated using radiation therapy along with chemotherapy, radical hysterectomy and lymphadenectomy. Stage III and IVA invasive carcinomas (Table 2) are treated using a combination of radiation therapy and chemotherapy, notably cisplatin, whereas for Stage IVB, patients can not be treated using radiation or surgery, chemotherapy is proposed for palliation and novel drug combinations may be investigated (NCI, 2003b).

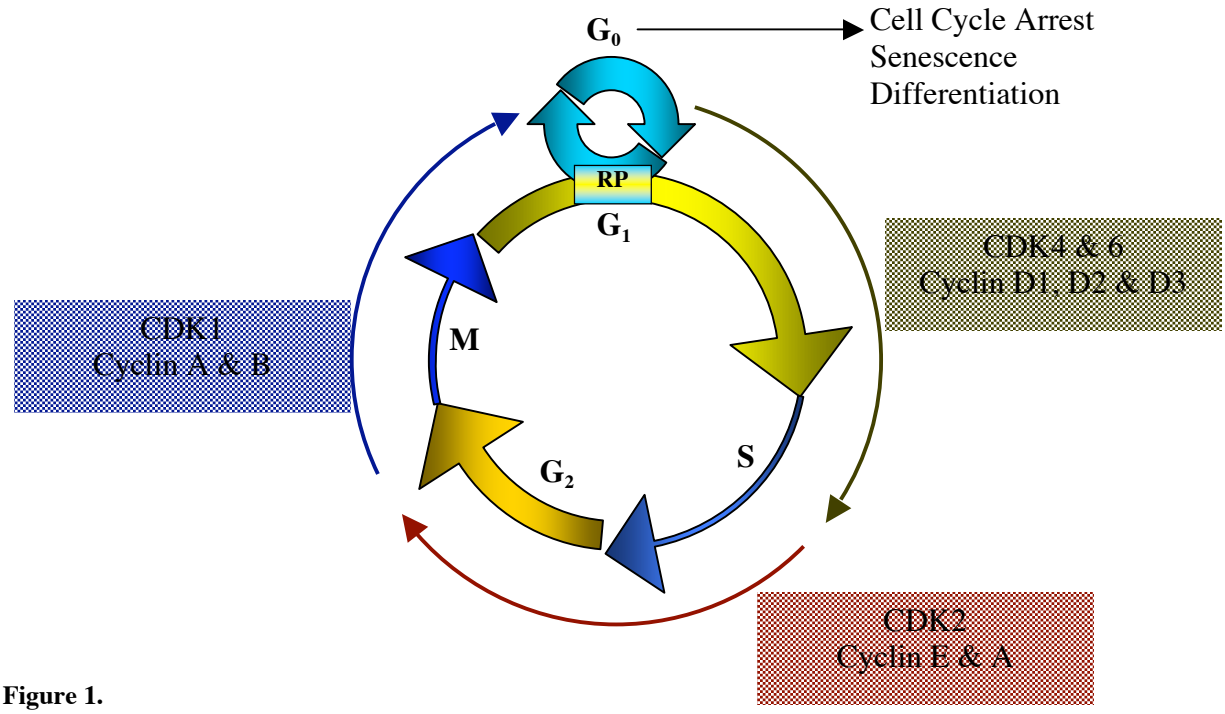


Figure 1.
The cell cycle.
Cellular division requires passage through the four phases of the cell cycle: Gap 1 (G_1 phase), DNA synthesis (S phase), Gap 2 (G_2 phase) and mitosis (M phase). Non-dividing cells exit the cell cycle and enter the G_0 phase. The cell cycle is controlled by phase-specific Cyclin Dependant Kinases (CDK) and cyclins.
Adapted from Ivanchuk and Rutka 2004.

2. Cancer: role of the cell cycle and apoptosis

2.1 The cell cycle

Cancer is a generic term used to describe malignant neoplasms and fundamentally this is achieved by deregulating cell proliferation and suppressing programmed cell death. Cell proliferation is dictated by a process called the cell cycle that describes the successive events of genome replication (Synthesis or S-phase), segregation of these two copies of the genome and division into two daughter cells (Mitosis or M-phase) (Fig. 1). Between these are the Gap 1 and Gap 2 phases (G_1 and G_2, respectively). In early G_1, cells commit to division at the restriction point (RP) and continue into late G_1 in which they grow and prepare for chromosomal replication in the S-phase. However, in the absence of mitogenic or the presence of anti-mitogenic signals, they go into quiescence or G_0, effectively exiting the cell cycle and in doing so halting proliferation (Ivanchuk and Rutka, 2004). Control of the cell cycle takes the form of checkpoints at the boundaries of the different phases. In normal cells, these monitor the integrity of the cell cycle and, where errors are detected, interrupt the course of the cell cycle for these to be repaired. Transition from phase to phase is organised by different combinations of cyclins and cyclin-dependant kinases (CDKs) (Lee and Yang, 2003). On their own, CDKs are not active and require the association of cyclins, their regulatory sub-unit (Fig. 2). CDK/cyclin association is regulated by phosphorylation. CDKs are mono-phosphorylated by CDK activating kinases which induces a conformational change in the protein, allowing cyclin association. On the other hand, extensive phosphorylation by de-activating kinases induces further conformational change, precluding cyclin association (Fig. 2) (Ivanchuk and Rutka, 2004). Evans *et al.* first discovered cyclins in 1982 and their cyclic expression gave rise to their name (Evans et al., 1983) and indicated that proteolysis was an important part of their regulation (Ivanchuk and Rutka, 2004). This has since been explored in greater detail and degradation of cyclins by the 26S proteasome is indeed an intrinsic component of their control. Degradation by the 26S proteasome is a common feature in the regulation of proteins implicated in the cell cycle and is discussed in a following section.

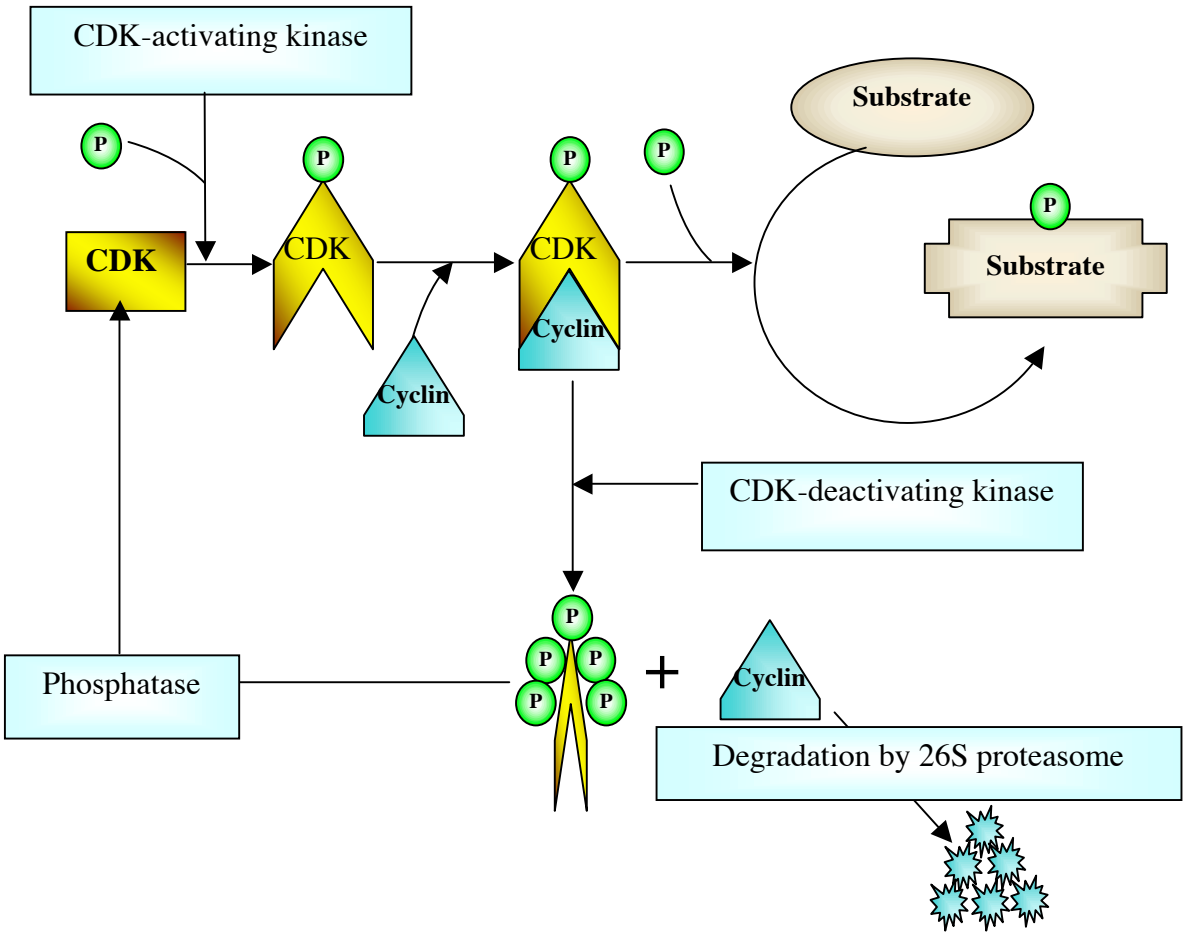


Figure 2.
Regulation of cyclin-dependant kinases (CDK).
CDK-activating kinase phosphorylates CDKs, inducing a conformational change and thus permitting cyclin binding. The CDK-cyclin is an active kinase complex that has specific targets depending on the CDK and cyclin combination. CDK-cyclin kinases are inactivated by further phosphorylation that induces additional conformational change and release of the cyclin. Free cyclin is then degraded by proteolysis in the 26S proteasome. Phosphatases act on the extensively phosphorylated CDK that is then capable of being activated once again by a CDK-activating kinase.
Adapted from Ivanchuk and Rutka 2004.

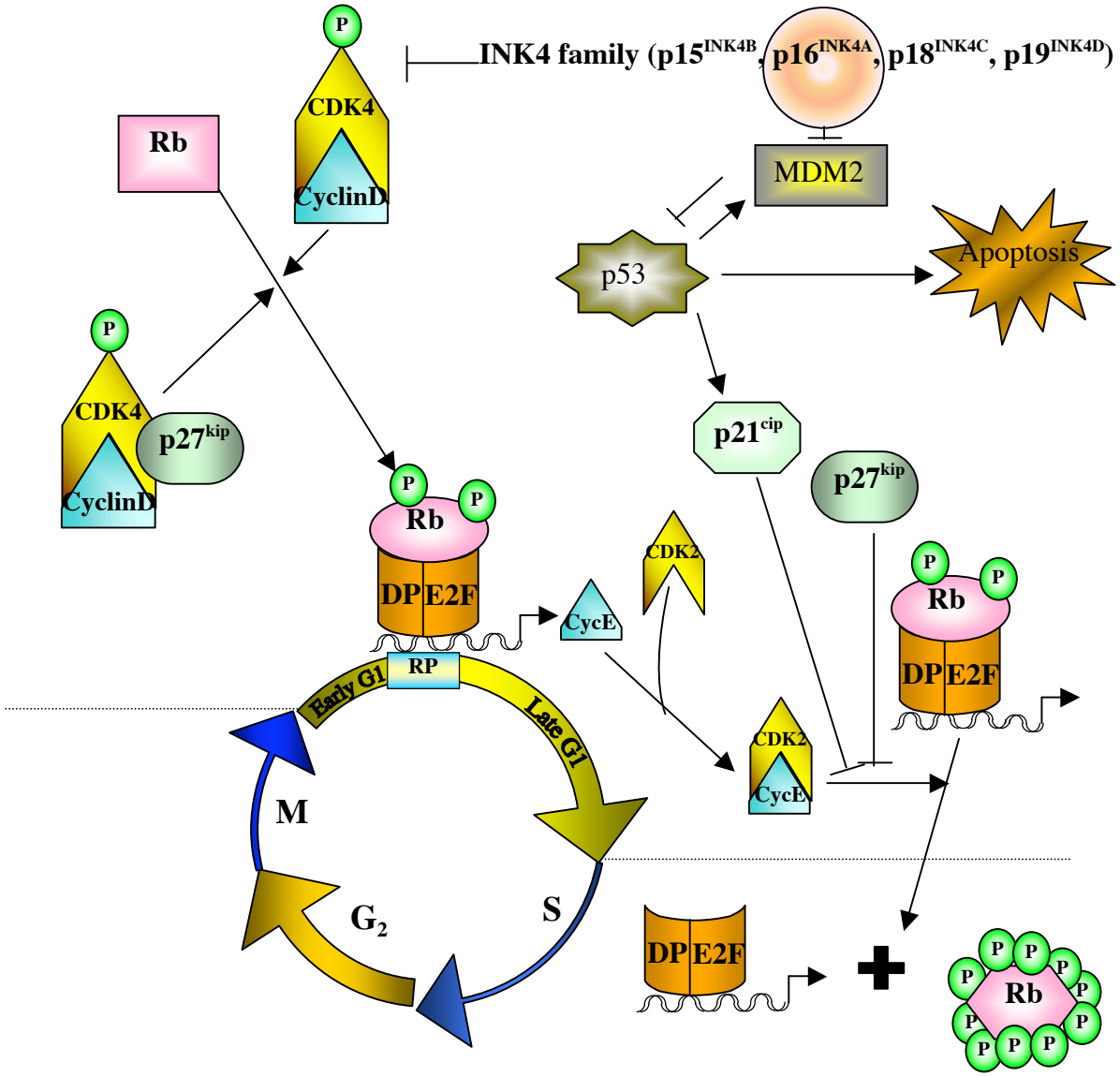
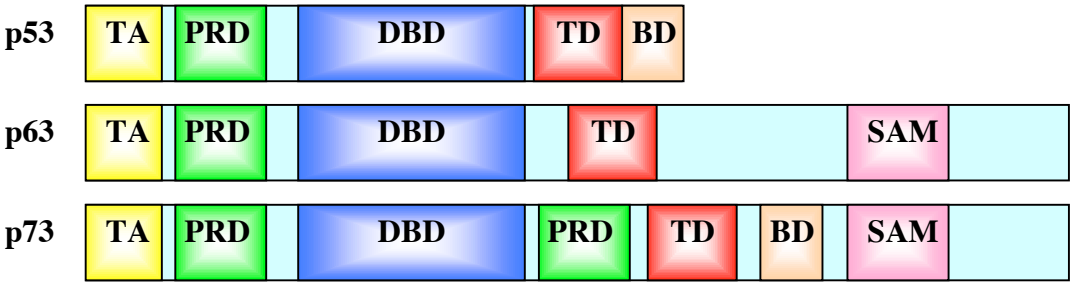


Figure 3.
Roles of pRb and p53 in G_1 to S-phase checkpoint.
G_1 is divided into Early G_1 prior to RP and Late G_1 post RP. The INK4 family of proteins and p27 regulate CDK4-Cyclin D by binding to the CDKs. This complex partially phosphorylates Rb and this hypophosphorylated Rb species activates DP-E2F transcription factor mediated expression of Cyclin E. As CDK4-Cyclin D titrates p27 away from CDK2-Cyclin E, the later extensively phosphorylates Rb which dissociates from DP-E2F transcription factor allowing expression of additional genes and transition into S-Phase.
Adapted from DeWolfe and Gaston 2004 and Ivanchuk and Rutka 2004.

A



B

	Amino acid sequence homology			
	TA	**DBD**	**TD**	**SAM**
p73 vs p63	37%	91%	74%	83%
p73 vs p53	30%	79%	33%	N/A
p63 vs p53	26%	80%	31%	N/A

Figure 4.
Functional domains of the p53 family proteins.
The p53 family is composed of three proteins which have homologous functional domains: Trans-Activation domain (TA), Proline Rich Domain (PRD) also known as Src Homology 3 domain (SH3), DNA-Binding Domain (DBD), Tetramization Domain (TD). P63 and p73 have C-terminal extensions which englobe a Sterile-α-Motif (SAM) domain whereas p53 and p73 share a Basic Domain (BD). Amino acid homology between these proteins is also shown.
Adapted from Harms et al. 2004 and Melino et al. 2003.

2.2 Rb and the p53 family

Rb protein, the product of the retinoblastoma-susceptibility gene, and the tumour suppresser protein p53 are highly important in the regulation of the cell cycle and mediation of cell death. They have also been found to be highly important in cancer and are well-documented proto-oncoproteins. Indeed, both Rb and p53 proteins are the most frequently genetically- or functionally compromised proteins in cancer (Chau and Wang, 2003; Haupt et al., 2003; Ivanchuk and Rutka, 2004; Levine, 1997; Nakamura, 2004). In the cell cycle, these two proteins act in mid G_1 phase at the Restriction Point (Fig. 3). This point represents a critical instant of the cell cycle where comitance to division is made and represents the G_1 to S-phase checkpoint (DeWolf and Gaston, 2004). In early G_1, Rb is bound to the transcription factor complex E2F-DP inactivating it. In the presence of sufficient cyclin D, the CDK4-cyclin D complex is formed which phosphorylates Rb only at certain sites. This limited phosphorylation of Rb partially activates the E2F-DP transcription factor complex, initiating the expression of genes encoding cyclin E and the additional genes required for G_1 to S-phase transition. CDK2-cyclin E phosphorylates additional sites on Rb, rendering it completely inactive and inducing the phase transition. This system is subjected to regulation from the INK4 family proteins (Fig. 3) which exert their effect through interacting directly with the CDK4 and 6 (Fig. 2 and 3).

Cellular stress, including DNA damage and oxidative stress (hypoxia), activate the proteins of the p53 transcription factor family. These integrate cellular response by regulating the expression of target genes. The p53 family is composed of the transcription factor proteins p53, p63 and p73 schematically represented in Fig. 4. These proteins activate the transcription of many of the same genes. For example: p21, a protein involved in repression of the cell cycle as previously described, and Bax, a protein involved in apoptosis (programmed cell death). However, both p73 and p63 are fundamentally different to p53 owing to their extended C-terminal domain and to the existence of multiple splice- and initiation-variants (Melino, 2003). Indeed, the p53-family proteins share sequence homology in their TransActivating (TA) domains, DNA-Binding Domains (DBD) and Tetramization Domains (TD) (Fig. 4) and all have a proline-rich (PR or SH3) domain (p73 has two). On the other hand, p63 lacks the C-terminal Basic Domain (BD) of p73 and p53 lacks the C-terminal Sterile-α-Motif (SAM) domain of the p63 and p73 proteins (Harms et al., 2004; Melino, 2003). Additionally, whereas p53 predominantly exists as a single isoform, p63 and p73 have three and seven splice-variants respectively and transcription of each of these splice-variants

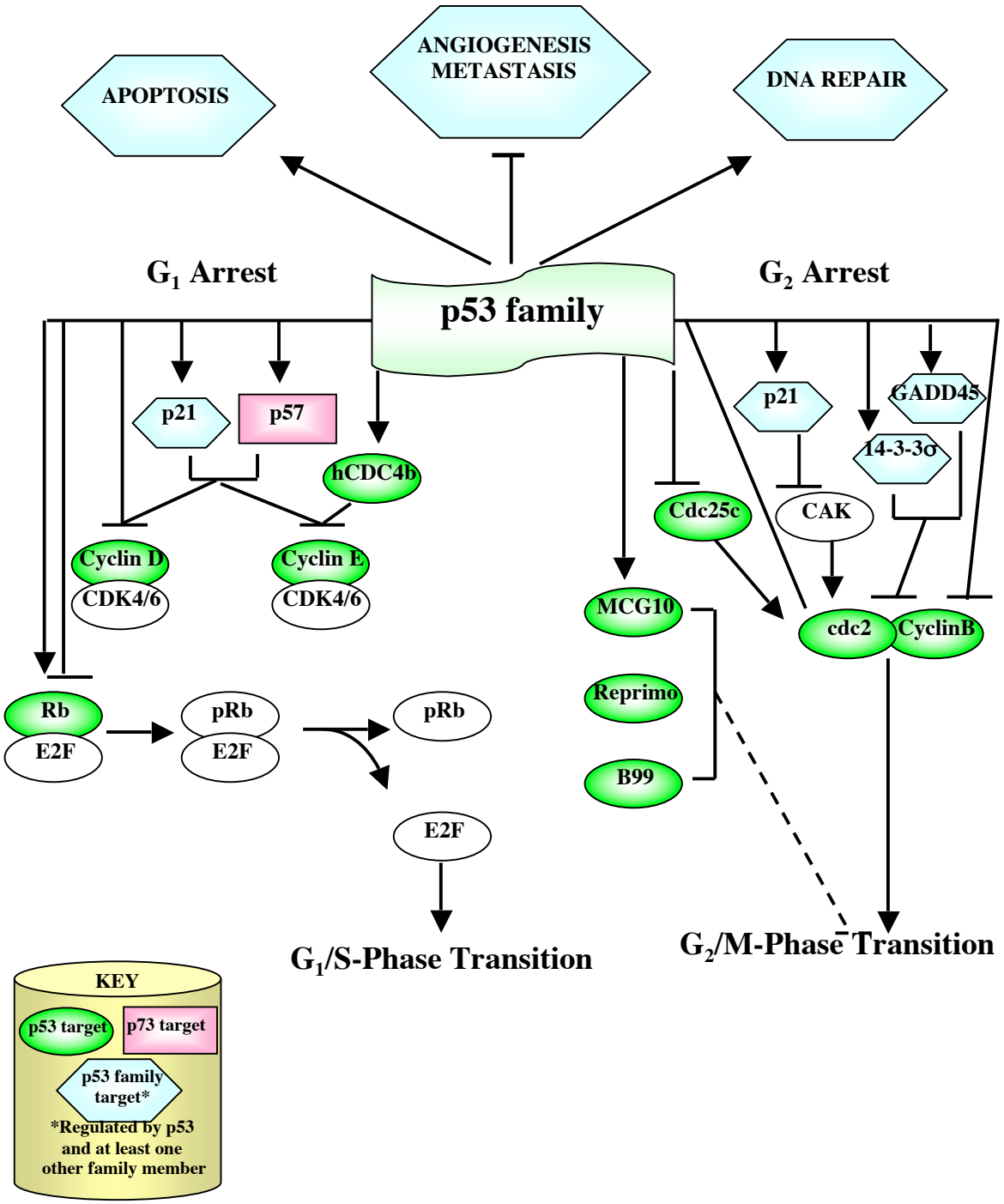


Figure 5.
The p53 protein family regulate the expression of various genes.
The genes regulated by the p53 family are involved in many pathways implicated in cell growth, cell cycle control, apoptosis and DNA repair.
Adapted from Harms et al. 2004.

can be initiated from either the major upstream promoter or a promoter situated in exon 3. The proteins resulting from the second promoter are called the TA-variants as they are abrogated in the TransActivating domain (Harms et al., 2004; Westfall and Pietenpol, 2004).

As a family, p53, p63 and p73 regulate the transcription of many genes implicated in several pathways all concerned with the integrity of normal cellular function (Fig. 5). In the case of cell cycle arrest, the principal gene upregulated by all proteins of the p53 family is p21 which is involved in both G_1 to S-phase and G_2 to M-phase arrest. However, other genes are also regulated in order to act at all the levels of regulation (Harms et al., 2004). The p53 family also directs the DNA repair pathways, represses angiogenesis and metastasis and also induces programmed cell death or apoptosis. There are two well-described pathways for apoptosis named extrinsic and intrinsic (Fig. 5). The extrinsic pathway integrates extracellular signals to activate the "initiator" caspase 8, whereas the intrinsic pathway creates release of cytochrome C from the mithochondria, activating the "initiator" caspase 9. These "initiator" caspases converge onto the "effector" caspases 3, 6 and 7 that trigger cellular events, culminating in apoptosis. Activation of the p53 family of proteins involves a complex interplay of post-translational modification by phosphorylation, acetylation and ubiquitination. The p53 family of proteins also regulate their own expression and the proteins involved in their regulation (Harms et al., 2004).

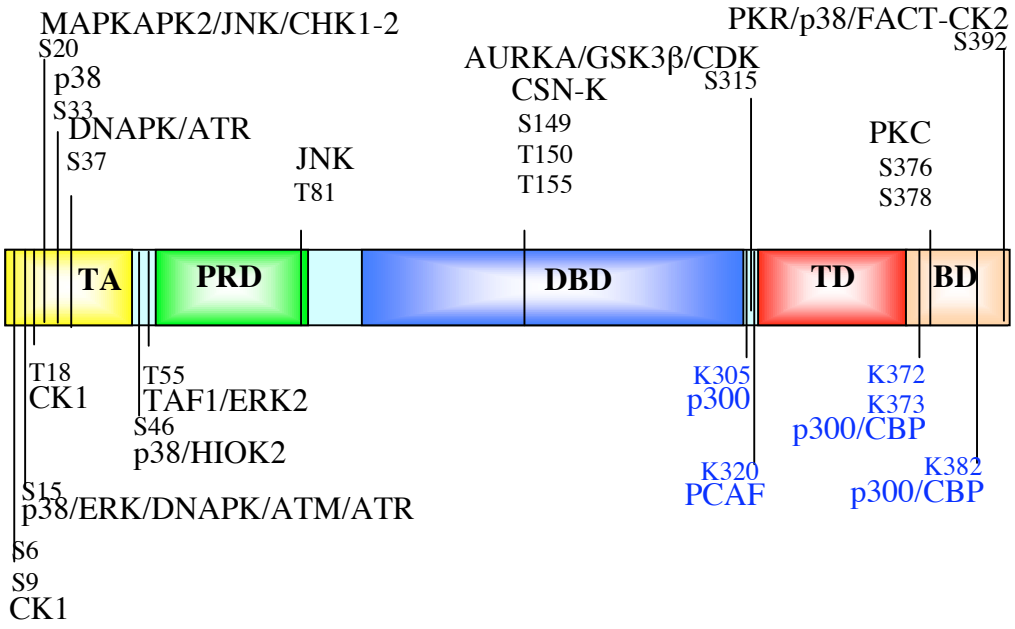


Figure 6.
Schematic representation of post-translational phosphorylation and acetylation of p53 protein.
The schematic representation of p53 protein is annotated with the enzymes responsible for phosphorylation (black) and acetylation (blue). FACT is a heterodimer of SPT16 and SRP1. PKR is a double-stranded RNA-activated kinase. TAF1 is a component of the transcription factor TFIID. ATM, ataxia telangictasia mutated; ATR, ataxia telangiectasia and Rad3 related protein; AURKA, aurora kinase A; CDK, cyclin-dependant kinase; CHK, checkpoint kinase; CK, casein kinase; CSN-K, COP9 signalosome associated kinase complex; DNAPK, DNA-dependant protein kinase; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase-3β; HIPK2, homeodomain-interacting protein kinase 2; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor.
Adapted from Bode and Dong 2004.

The most well-documented protein of the p53 family is p53 itself as it has benefited from over 20 years of research whereas p63 and p73 where described only relatively recently in 1998 and 1997 respectively (Westfall and Pietenpol, 2004). p53 is, as is p63 and p73, regulated by the result of an extremely complex interplay of site-specific acetylation, phosphorylation and ubiquitination (Fig. 6). Activation by phosphorylation or acetylation stabilises p53, induces tetramerization via its TD. This induces its translocation to the nucleus where it interacts with basic transcription factors such as hTAFII-31 and transcriptional co-activators p300/cAMP Response-Element Binding-Binding Protein (p300/CREB-BP or p300/CBP) and PCAF (p300/CBP-Associated Factor) via its N-terminal domain. This initiates the transcription of specific genes as the DBD of p53 recognises p53 response elements in the genome. The expression of specific genes depends on the signal that initiated the p53 response. For example, in unstimulated cells, p53 is phosphorylated at Ser 9, 15, 20 and 372 during G1, Ser 37 during S-phase and both Ser 37 and 392 during G_2-M-phase. Another example is a response to UV light which induces the phosphorylation of Ser 46 by the Homeodomain-Interacting Protein Kinase 2 (HIPK2). This increases acetylation of p53 by p300/CBP at Lys 382 and in turn specifically induces the expression of the pro-apoptotic gene p53AIP1 (Hofmann et al., 2002). It is possible that heterogeneous pools of p53 may co-exist, integrating parallel cellular pathways at the same time. There are various sites of phosphorylation and acetylation on p53, however, the majority of phosphorylation occurs in the N-terminal domain whereas the greater part of acetylation occurs in the C-terminal domain (Fig. 6). Covalent attachment of ubiquitin, ubiquitination, is another post-translational modification involved in the regulation of p53. In normal cells, this has been demonstrated to be performed by MDM2 (Mouse Double-Minute 2) and, more recently, Pirh2 (Leng et al., 2003), COP1 (Constitutively photomorphogenic 1) (Dornan et al., 2004b) and Topors (Rajendra et al., 2004).

The *mdm2* gene was first identified on double-minute chromosomes of spontaneously transformed mouse 3T3 fibroblasts, and subsequently the mdm2 protein was found to be associated with p53 protein (Moll and Petrenko, 2003). It is important at this point to note the nomenclature used freely in the literature for this protein and its encoding gene. The *mdm2* gene and its protein product mdm2 were isolated in mice and the finding of the homologous human gene and protein product have given rise to the use of "hdm2" or "HDM2" to describe these. However, some authors use the terms *MDM2* and MDM2 in reference to the human set in order to distinguish between species yet, in the literature, mdm2 and hdm2 are used interchangeably and distinction is rarely made. In general, references in the literature are made to the human variety, unless specified by the authors. In the proceedings of this text and in accordance with this nomenclature, any reference to MDM2 will refer to the human type.

MDM2 requires a primary binding site in the p53 N-terminal domain spanning the region ^{18}TFSDLWKLLP^{27} (Picksley et al., 1994) and phosphorylation of Ser 20 (by CHeckpoint Kinase 1, CHK1) is pivotal in inhibiting MDM2 interaction, resulting in inhibition of ubiquitination of p53. Poly-ubiquitination may lead to 26 proteasome-mediated degradation or modified cellular localisation of p53 protein. Although p73 binds MDM2 and p300/CBP (Melino, 2003), MDM2 exerts its effect by titrating p73, inhibiting p300/CBP binding rather than inducing its ubiquitination and degradation (Balint et al., 1999). p63 also interacts with MDM2, however, the function of this remains controversial as contradictory results have been obtained independently by two groups (Calabro et al., 2002; Little and Jochemsen, 2001). Melino and co-workers suggest that this may be due to the difference in the cell lines used in the two studies as regulatory proteins are cell-type specific (Melino, 2003).

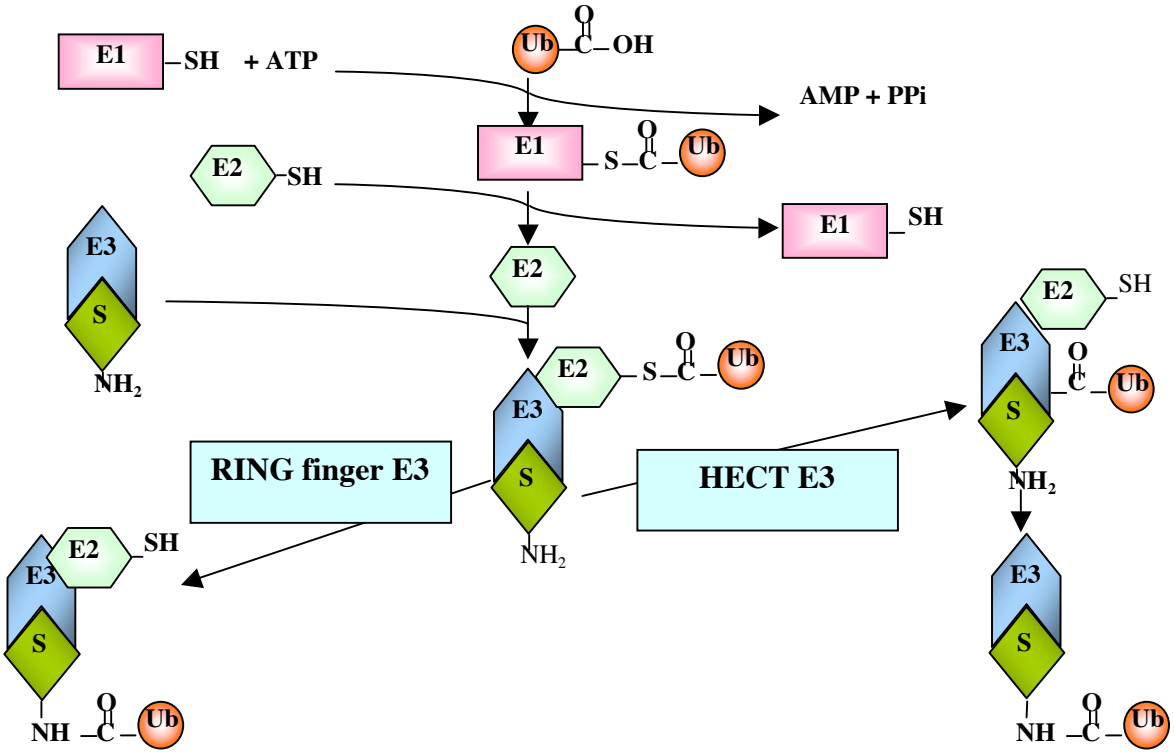


Figure 7.
The ubiquitination cascade.
Free Ub is activated by formation of a thiol-ester between the E1 Ub-activating enzyme and the C-terminal glycine residue of Ub. Ub is then transferred to one of the E2 Ub-conjugating enzymes (32 examples exist in mammalian cells). E2-Ub then associates with E3 Ub-ligase enzymes. For HECT domain E3s, Ub is transferred to the cysteine residue in its active site and then to the substrate, whereas RING finger E3s mediate transfer directly from the E2 to the substrate.
Adapted from Fang and Weissman 2004.

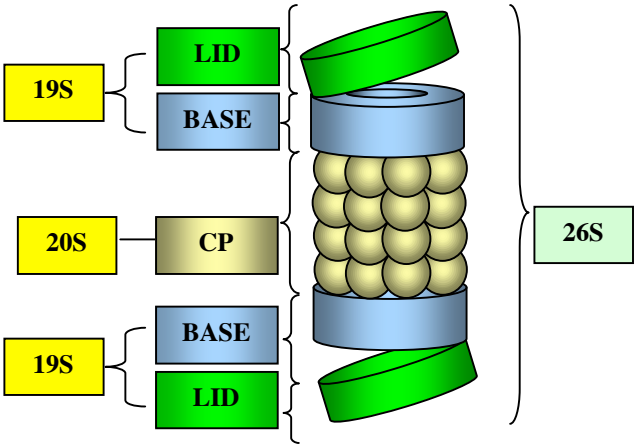


Figure 8.
The 26S proteasome.
The 26S proteasome is composed of two 19S regulatory domains and one 20S proteolytic domain. The 19S regulatory domain is composed of a lid and a base. Poly-ubiquitinated substrates are recognized by the lid, unfolded by the base and passed into the proteolytic 20S core domain.
Adapted from Li and Deng 2003.

p53 is of high importance in human cancer. This is underlined by the 18,000 mutations (Bode and Dong, 2004) that are associated with 50% of human cancers and the functional inactivation of p53 which is associated with a further 20% of these (Harms et al., 2004; Melino, 2003). However, although the p63 and p73 are part of the p53 family that is centrally important for maintaining cellular integrity, mutations in these proteins are rare and impaired function of these is not associated with human cancer.

2.3 Degradation of the cell cycle regulatory proteins

As highlighted earlier, degradation is a key event in the regulation of the proteins that control the cell cycle. The most important system that has been described for this degradation is the ubiquitin-26S proteasome system. In this system, substrates destined for degradation are "tagged" by covalent attachment of multiple ubiquitin (Ub) molecules which are then targeted to the 26S proteasome that mediates proteolysis (Bajorek and Glickman, 2004). Ubiquitin is a highly conserved polypeptide consisting of 76 amino acids, and rather aptly, it is ubiquitously expressed in eukaryotes. Substrate ubiquitination is mediated by sequential action of three enzymes: E1, Ub-activating; E2, Ub-conjugating and E3; Ub-protein ligase. The E1 Ub-activating protein binds Ub moieties by a thiol-ester bond between a cysteine and the C-terminal glycine residue of Ub. Transesterification passes Ub moieties from E1 Ub-activating to E2 Ub-conjugating enzyme that subsequently interacts with the E3 Ub-ligase. This complex binds substrates via the E3 Ub-ligase and transfers the Ub moiety to an ε-amino group of a lysine residue of either the substrate or to a Ub moiety previously attached to the substrate (Fig. 7) (Fang and Weissman, 2004). Mono-ubiquitination is insufficient to induce degradation, yet it serves a multitude of functions including protein trafficking (Yang et al., 2004). Degradation, on the other hand, occurs when four or more Ub moieties are attached to a protein (poly-ubiquitination), the first through a lysine residue of the substrate and subsequently through the lysine number 48 of the Ub moieties. Substrate specificity is largely dictated by the E3 Ub-ligase and it is predicted that over 300 examples of the E3 Ub-ligase may exist in human cells. On the other hand, there is only one E1 Ub-activating enzyme and approximately 30 E2 Ub-conjugating enzymes in eukaryotes. E3 Ub-ligases have been predicted from databases yet the two most studied are those containing the Homologous to E6-AP Carboxyl Terminus (HECT) domain and the Really Interesting New Gene (RING)

finger domain. These differ fundamentally in the mechanism used to transfer Ub to the substrate protein. Indeed, whereas RING finger domain E3 Ub-ligases mediate the transfer of the Ub moiety directly from the E2 Ub-conjugating enzyme to the substrate, HECT domain E3 Ub-ligases form an transient intermediate species in which the Ub moiety is covalently attached to it (Fig. 7) (Fang and Weissman, 2004).

Poly-ubiquitinated substrates are directed to the 26S proteasome. The eukaryotic 26S proteasome is 2.5MDa multi-protein (32 different subunits) multi-substrate protease (Bajorek and Glickman, 2004). This structure can be dissociated into two stable 19S and 20S complexes (Fig. 8). Proteolysis takes place in the tubular 20S structure that, in the absence of the 19S regulatory complex, is autoinactivated. The 19S regulatory complex is composed of a base, a lid and associated proteins (Fig. 8). Poly-ubiquitinated proteins are recognised by the 19S regulatory complex in a poorly described mechanism. However, it has been reported that this extended 19S complex displays chaperone-like and ATPase activity. Additionally, the subunits constituting the 19S have affinity for polyubiquitin chains and that they have de-ubiquitination activity, which has been demonstrated to be a prerequisite of proteolysis (Bajorek and Glickman, 2004; Hartmann-Petersen et al., 2003).

Cyclins undergo Ub-mediated degradation and their poly-ubiquitination is mediated by two protein complexes, both containing a multi-subunit RING finger E3 Ub-ligase. Indeed, G_1 cyclins are poly-ubiquitinated by the Skp1-Cul-F-box (SCF) complex whereas M-phase cyclins are poly-ubiquitinated by the Anaphase-Promoting Complex (APC). The activity of APC is regulated by phosphorylation in function of the cell cycle and does not degrade phosphorylated substrates whereas the SCF complex is constitutively active and degrades only phosphorylated substrates (Murray, 2004). Additionally, the degradation of p27 is also induced by poly-ubiquitination by the SCF complex (Morimoto et al., 2000).

As described above, p53 is also regulated by poly-ubiquitination and subsequent degradation by the 26S proteasome (Yang et al., 2004). p53 levels are tightly regulated by an autoregulatory feedback loop that it constructs with the protein MDM2. MDM2 is a 90KDa single subunit RING finger domain E3 Ub-ligase and is itself an oncoprotein (it is over-expressed in more than 60% of human breast cancers) (Deb, 2003). MDM2 binds p53 through an interaction between its N-terminal hydrophobic groove in which the N-terminal p53 residues (18-26) bind (Fig. 9). The RING finger domain is situated near the C-terminus of

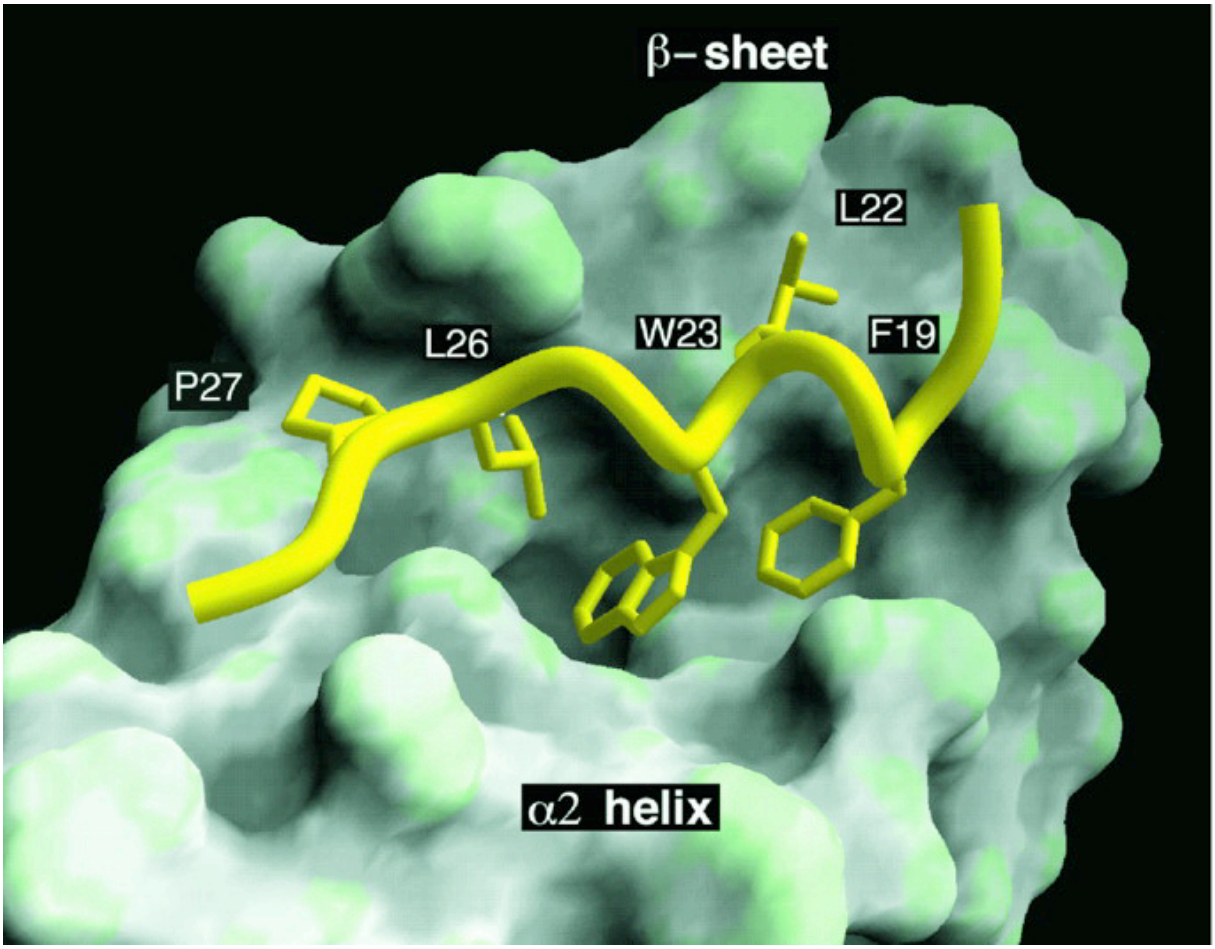


Figure 9.
Interaction of MDM2 and p53.
The MDM2 hydrophobic pocket (gray) into which Phe 19, Trp 23 and Leu 26 of the p53-derived peptide inserts (yellow).
Adapted from Kussie et al. 1996.

MDM2 that mediates p53 ubiquitination, mainly in its C-terminal domain (Moll and Petrenko, 2003). Poly-ubiquitination of p53, which is required for 26S proteasome-mediated degradation, is brought about in the nucleus by a tertiary complex it forms with p53 and p300/CBP. It has been proposed that p300/CBP is a novel "E4" Ub-ligase as, in the absence of CBP, MDM2 only mono-ubiquitinates p53 (Grossman et al., 2003) and that a MDM2 mutant defective in p300-binding ubiquitinates p53 yet does not induce degradation of p53 (Zhu et al., 2001). In agreement with this hypothesis, non-proteasomal functions of substrate mono-ubiquitination have been documented (Fang and Weissman, 2004; Moll and Petrenko, 2003) and both the SCF complex and the APC may also have intrinsic E4 Ub-ligase activity as these are multi-protein structures whereas MDM2 is a single subunit E3 Ub-ligase. Once poly-ubiquitinated, p53 is degraded by the proteasome and it is proposed that the Rad23 protein, associated with the 19S subunit complex of the 26S proteasome is responsible for interacting with this poly-ubiquitin chain and de-ubiquitinating p53 before it is degraded (Hartmann-Petersen et al., 2003).

The Ub-26S proteasome system degrades proteins both rapidly and efficiently. For example, the half-life of p53 in the cell is reduced from hours to minutes by this system (Yang et al., 2004). However, there exists an Ub-independent pathway leading to 26S proteasome degradation (Hoyt and Coffino, 2004). Indeed, work performed by Philip Coffino's group on the Ornithine DeCarboxylase (ODC) and its regulatory protein Antizyme has revealed a C-terminal sequence of ODC effectively mimics poly-ubiquitin chains. This phenomenon is regulated by the N-terminus of the Antizyme protein and results in recognition of the poly-Ub-mimicking peptide sequence of ODC by the 26S proteasome and the subsequent degradation of the protein. This process has been demonstrated to be wholly independent of ubiquitination (Hoyt and Coffino, 2004). Interestingly, Philip Coffino's group has also described other proteins that have been reported as having a "degradation domain" capable of replacing that of ODC, notably cyclin B (Li et al., 1996), and p53 (Li and Coffino, 1996b). Whereas ODC is naturally degraded independently of Ub, both cyclin B and p53, as discussed previously, are also under the control of Ub-mediated degradation. The precise nature and the interplay of these two, possibly complementary, systems has yet to be elucidated. Other proteins such as the CDK inhibitor p21 implicated in cell cycle regulation have been demonstrated to be degraded in both Ub-dependant and independent pathways. However, this protein contains a "degradation domain" in its C-terminus that is a binding site for C8 alpha subunit of 20S proteasome (Touitou et al., 2001). For degradation in the 20S proteasome complex, proteins are first required to pass through the 19S subunit complex. In the case of ODC, the degradation domain targets the 19S extended complex, indicating a possible fundamental difference in the degradation pathways of ODC and p21. It has been proposed that, to target the 19S complex, p21 adopts a non-native conformation (Hoyt and Coffino, 2004).

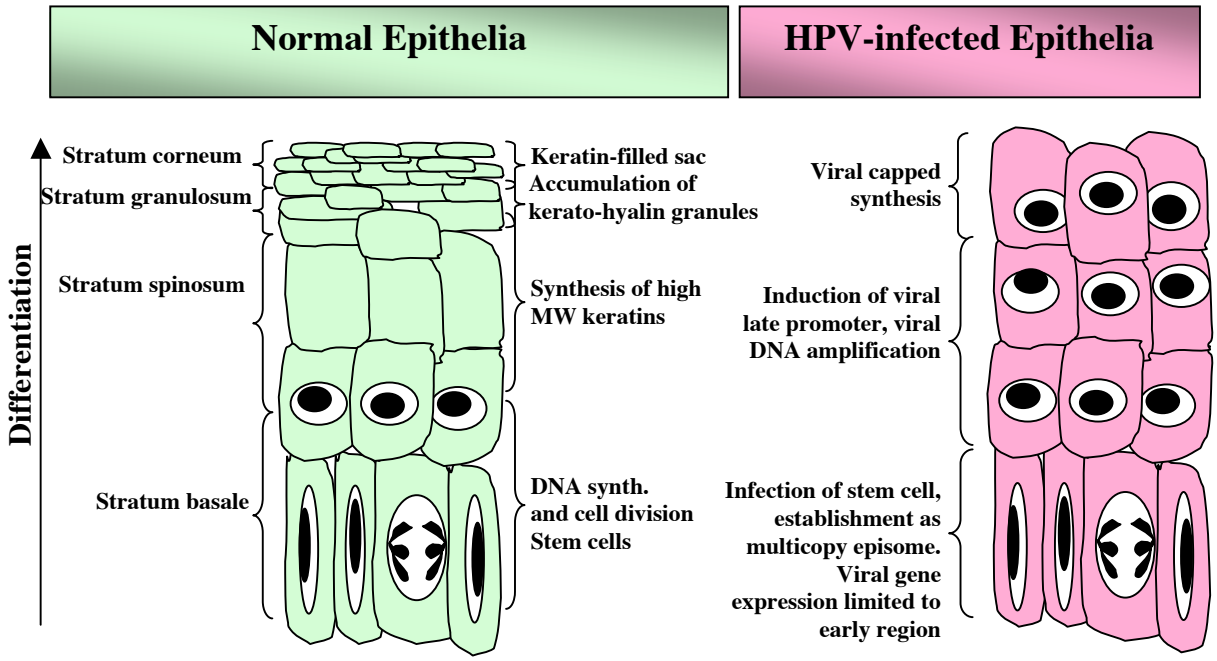


Figure 10.
Comparison of normal and HPV-infected epithelium.
Whereas normal keratinocytes progressively loose their nuclei an become essentially composed of keratin at the stratum corneum, HPV-infected cells maintain their nuclei and as the move up from the basal layer differentiation signals induce expression from the major late promoter and virus production is initiated. Viral particles are released from the uppermost layers (not shown).
Adapted from Longworth and Laimins 2004 and Stubenrauch and Laimins 1999.

3. HPV and cancer

3.1 HPV life cycle in epithelial cells

HPVs infect the dividing cells of the epithelium basal layer that is thought to be permitted by microwounds or abrasion of the superior layers. Basal layer cells which are stem cells and the supra-basal transit-amplifying cells divide continuously. Daughter cells either remain in the basal layer or migrate through the suprabasal regions. The latter are committed to differentiation and exit the cell cycle. Differentiated epithelial cells lack nuclei and become increasingly rich in keratin, whereas HPV-infected cells maintain nuclei and modulate the cytokeratin matrix (Fig. 10) (Blachon and Demeret, 2003; Longworth and Laimins, 2004b; Stubenrauch and Laimins, 1999). In non-differentiated basal cells the HPV DNA establishes itself in the nucleus as a low-copy extrachromosomal element or episome. Although in this latent phase there is moderate expression of early gene products that may enhance proliferation at the basal layer (zur Hausen, 2002) and are required to maintain episomal DNA (Oh et al., 2004b), gene expression is largely suppressed. Differentiation of HPV-infected basal cells is determinant for the induction of productive life cycle. Indeed, terminally differentiated cells express viral genes and move to a rolling circle model of DNA replication, amplifying DNA to a high copy number and subsequently progeny virus is produced. It has been proposed that the differentiation-dependence of the productive HPV life cycle has been associated to unidentified cellular factors that are specific to differentiation (Longworth and Laimins, 2004b).

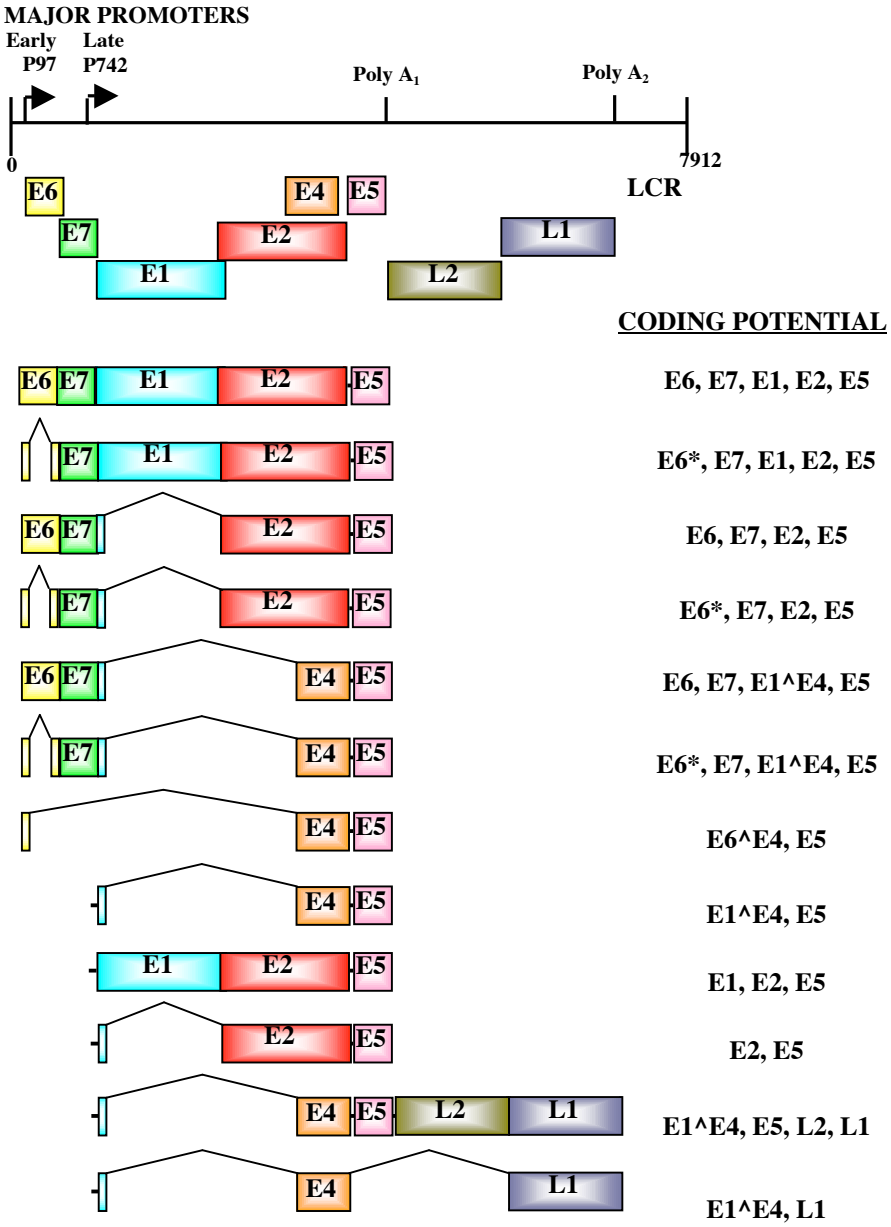


Figure 11.
Genome organisation and transcripts from the high-risk HPV type 31.
Transcripts initiated at the major early promoter are expressed in the basal layers of infected epithelia. Differentiation induces initiation of the major late promoter.
Adapted from Stubenrauch and Laimins 1999.

3.1.1 Genomic arrangement and protein products of HPVs

The HPV genome is a double stranded circular DNA of approximately 7900bp (exact size is dependant on type and, by definition, subject to variation) (Fig. 11). For all HPV types, transcripts encoding viral gene product open reading frames (ORFs) are polycistronic. The two major promoters initiate expression of either early or late genes, in HPV 31 these are respectively designated p97 and p742 with respect to nucleotide number and major early and major late with respect to the HPV life cycle. The first is a constitutively active promoter whereas the second is differentiation-dependant and the exact location of these promoters may vary with type.

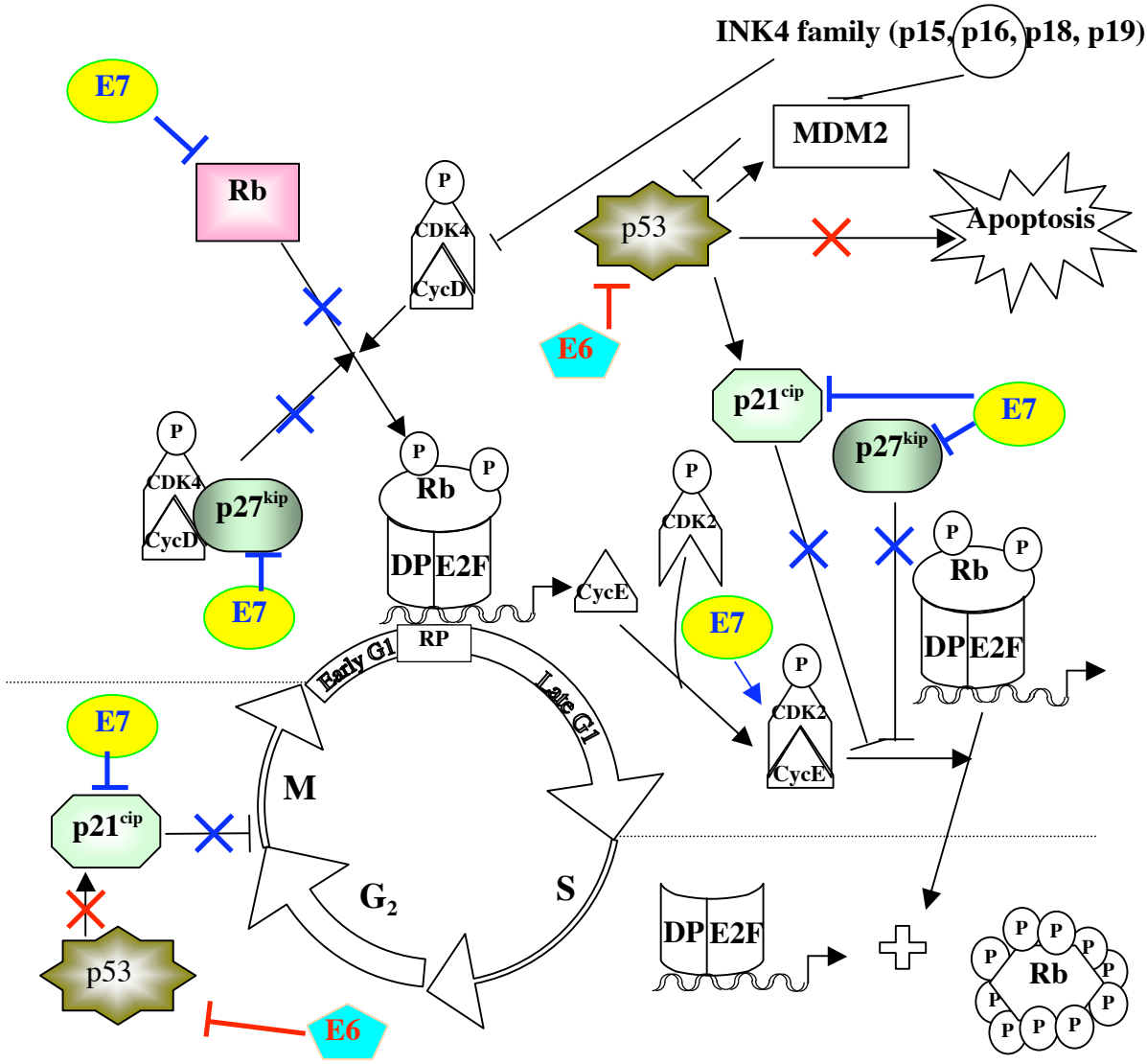


Figure 12.
Roles of E6 and E7 in the cell cycle.
E6 and E7 proteins directly interact with several proteins involved in cell cycle checkpoint maintenance and act to prohibit negative regulation of the cycle and apoptosis.

3.1.1.1 Early gene products

The first transcripts are from the constitutive or major early promoter and gene products include E1, E2, E5, E6 and E7 (Fig. 11). Gene products E6 and E7 of low- and high-risk HPV are important for maintenance of genomic episomal DNA (Oh et al., 2004b) and, by interacting with host cell factors controlling host cell cycle, preventing exit (Fig. 12) (Burd, 2003; Stubenrauch and Laimins, 1999). However, only in high-risk HPV do E6 and E7 respectively bind and degrade p53, pRb and Rb family proteins and Low-risk HPV E7 does bind pRb, but with a much less affinity (Munger et al., 1992). The degradation of p53 and the proteins of the Rb family is an important factor leading to tumourigenesis of high-risk HPVs. This is mediated by E6 and E7 proteins and will be discussed in greater detail in a proceeding section. However, the function of E6 and E7 gene products in the productive phase of the HPV life cycle is to subvert the cell-cycle regulation of terminally differentiated host cells, re-engaging the cell cycle and thus creating a favourable environment for viral replication.

The E2 gene product is a 50 KDa DNA-binding protein which can modulate E6 and E7 transcription by forming homodimers and recognising 12bp palindromic sequences of the LCR of genomic HPV DNA and subsequently the adjacent constitutive promoter (Stubenrauch and Laimins, 1999; Taylor et al., 2003). Additionally, E2 protein plays an essential role in replication of the viral genome. E2 protein interacts with the E1 gene product that is a 68KDa protein which shows helicase activities. These two proteins act together to induce and to mediate the replication of HPV genomic DNA.

There are three E2-binding elements surrounding the viral origin of replication (Taylor et al., 2003) and hexamers of E2 protein mediate recruitment of E1 protein. During the S-phase of the host cell-cycle, this complex initiates replication of the viral genome as episomes by recruiting cellular polymerases and accessory proteins such as the DNA polymerase-α and the Swi/Snf complex (Longworth and Laimins, 2004b; Taylor et al., 2003). Differentiation activates the productive HPV life cycle by a poorly understood system in which unknown cellular factors induce an expression from the differentiation-dependant or major late promoter (Longworth and Laimins, 2004b). Unlike the constitutive promoter, the differentiation-dependant promoter is not negatively controlled by E2, allowing high levels of expression of both E1 and E2 (Fig. 11), inducing amplification of the viral genome copy number (Steger and Corbach, 1997).

E4 and E5 transcripts are found in both transcripts from the constitutive and differentiation-dependant promoters, however, it is likely that the differentiation-dependant promoter is responsible for their expression (Glahder et al., 2003). E4 gene product is a 17KDa protein which is translated from the differentiation dependant major late promoter (Fig. 11). E4 ORF lacks the AUG initiation codon and uses the E1 sequence for initiation. Due to this, the E1^E4 protein product is derived from an alternative transcript splicing and contains the first five amino acids of E1 and the rest from the E4 ORF (Knight et al., 2004). E1^E4 is the most highly expressed HPV protein (Longworth and Laimins, 2004b) and in high-risk HPV16, E1^E4 expression coincides with DNA amplification. Association of high-risk E1^E4 with the cytokeratin networks of differentiating keratinocytes may cause the collapse of these structures. Additionally, E1^E4 of low- and high-risk HPVs (11 and 16) have been shown to induce G_2 cell cycle arrest (Davy et al., 2002), however, its effect on the HPV life cycle remains unclear.

E5 gene product is an 83 amino acid hydrophobic membrane protein localised in the Golgi apparatus, endoplasmic reticulum and nuclear membrane (Tsai and Chen, 2003). E5 is important in early infection, preventing DNA-damage induced apoptosis and stimulating cell growth by interacting with epidermal-growth-factor receptor, the platelet-derived growth-factor-1 receptor and the colony-stimulating factor-1 (zur Hausen, 2002). However, it has been found that (HPV16) E5 is not required for latent HPV infection but for the productive HPV life cycle. It has been postulated that it has a role in reprogramming differentiated cells to allow DNA synthesis involved in viral DNA amplification (Genther et al., 2003).

3.1.1.2 Late gene products

L1 and L2 gene products are proteins of 55 KDa and 75 KDa respectively. These structural proteins, required for virus encapsidation, are expressed in the most basaly-distant and terminally-differentiated epithelial cells. Virion assembly takes place in the nucleus and, following synthesis in the cytoplasm, L1 and L2 are translocated into the nucleus via intrinsic Nuclear Localisation Signals (NLSs). Subsequently, encapsidation of the double-stranded closed-circular and histone-associated HPV genome takes place (Touze et al., 2000). HPV infection is non-lytic, virions are released by shedding of epithelial squamae (Munger et al., 2004).

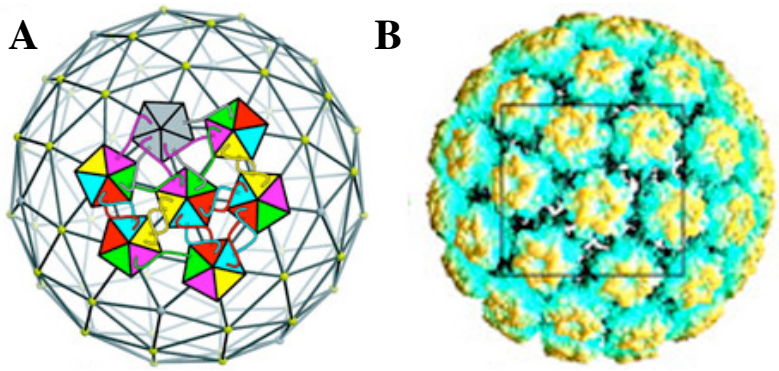


Figure 13.
HPV viral capsid.
(A) Schematic representation of icosahedral lattice made by the L1 and L2 proteins. This lattice is composed of 72 pentameric capsomers (grey), composed, themselves, of 5 copies of the major structural protein L1. (B) 3D representation of the capsomeres.
Adapted from Modis and Harrison 2002.

3.1.2 The HPV virion and infection of epithelial cells

Papillomavirus capsids are composed of 72 pentameric capsomers, each of these composed of 5 copies of the major structural protein L1 forming an icosahedral lattice (Fig. 13). L2, the minor structural protein, is present once for every 30 L1 copies, or more precisely, 12 copies per virion (Modis et al., 2002). Both structural proteins (L1 and L2) show DNA-binding activities (Touze et al., 2000; Zhou et al., 1994), yet L1 is sufficient to spontaneously form Virus-Like Particles (VLPs) which are capable of encapsidating reporter DNA. VLPs can be made entirely of L1 or both L1 and L2 proteins and are structurally similar to bona fide virions, exposing structural epitopes inciting the generation of antibodies (Touze et al., 2000). However, genuine papillomavirus virion capsids may be structurally more dynamic than VLPs. Indeed, it is proposed that for these two forms exist, a closed form for virions in solution and an open form induced by binding to cells which may in turn initiate internalisation and uncoating (Selinka et al., 2003). VLPs have aided the study of HPV, as authentic HPV virions are hard to produce in culture systems and only limited amounts have been isolated from natural lesions or murine models (Culp and Christensen, 2004).

One major area or research for which VLPs have been beneficial is in the study of viral entry into targeted epithelial cells. A model has been proposed in which a first, low-specificity binding step precedes specific binding to a receptor that mediates cellular internalisation of the HPV virion (Kawana et al., 1998) . It was, for some time, believed that the αVI integrin cell surface protein was required for HPV cell-binding and internalisation (Evander et al., 1997) but it seems that this is not the case (Sibbet et al., 2000). More recently, Shafti-Keraat *et al*. have demonstrated that the primary cell binding involves syndecan-1, a cell surface heparin sulphate proteoglycan. Interestingly, syndecan-1 expression is augmented in basal keratinocytes during wound healing and in migration of proliferating keratinocytes. The suprabasal microtrauma required for HPV infection of epithelial basal cells would therefore induce a highly favourable cellular environment for HPV binding to these cells. The interaction with syndecan-1 is through the L1 capsid protein and it is suggested that this is the primary HPV cell-binding incident (Shafti-Keramat et al., 2003). HPVs infect cells following the receptor-mediated, clathrin-dependant internalisation (Day et al., 2003) and it is suggested that this is mediated by the L2 structural protein (Selinka et al., 2003).

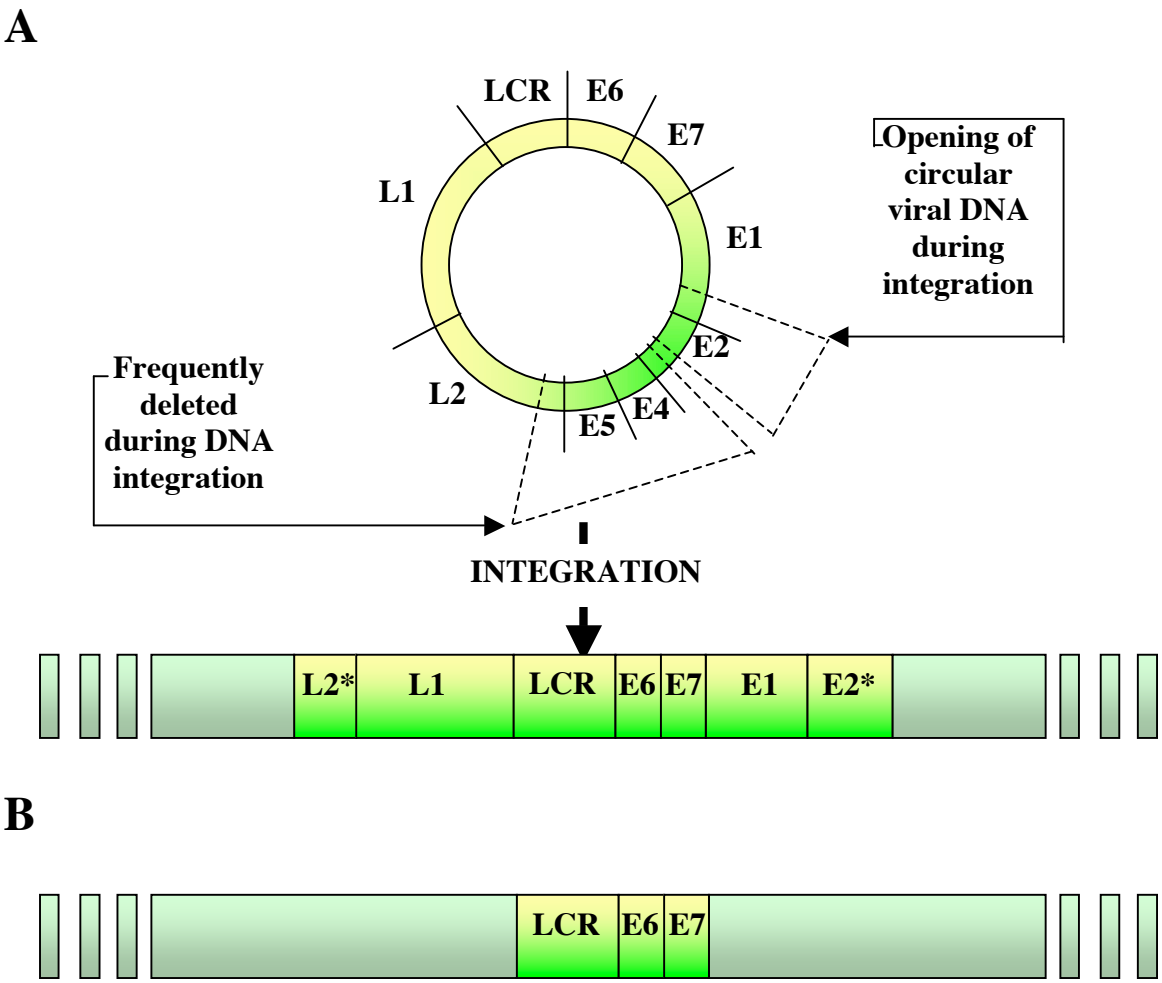


Figure 14.
Integration of the HPV DNA into host cell genome.
(A) HPV genomes consist of 7 to 8 Kbp and is divided into 8 open reading frames. Immortalization of cells is characterised by the integration of the HPV genome into the host DNA. Integration results in deletion of E2 and the adjacent E4, E5 and L2 open reading frames, prohibiting E2-regulation of transcription. (B) A minimal fragment is sufficient to immortalize cells.
Adapted from zur Hausen 2002 and Munger et al. 2004.

3.2 E6 and E7-mediated cellular immortalization

In benign HPV lesions, viral DNA is extrachromosomal whereas in high-grade SILs and cancers, HPV DNA is integrated into the host-cell genome. This integration (Fig. 14 A) disrupts or deletes the E2 ORF, abrogating E2 protein expression. Consequently, the constitutive promoter of viral genomic DNA is not repressed, increasing the expression of E6 and E7 genes. High-risk HPV E6 and E7 proteins can immortalize cultured human cells independently of each other, however, their affiliation increases efficacy (zur Hausen, 2002). Additionally, following integration into the host chromosome, other viral genes may be deleted or may not be transcribed (Munger et al., 2004) and a putative integration-dependant promoter has also been described (Glahder et al., 2003). Only the E6 and E7 genes are consistently expressed in cervical cancers which gives rise to a model in which only a minimal genome fragment is required to be maintained after integration (Fig. 14 B). Although E6 and E7 expression has been demonstrated to mediate both the latent phase of the HPV cell cycle and cellular immortalization, it has been observed that such cells are not tumourigenic and transformation of these cells requires additional mutations in the host-cell genome (Munger et al., 2004).

3.2.1 E7

3.2.1.1 The E7 protein

E7 gene product is a protein composed of 96 amino acids that form two domains (Fig. 15). The N-terminal 37 amino acids show homology to parts of the conserved regions 1 and 2 (CR1 and CR2) of adenovirus protein E1A and are named CR1H and CR2H respectively. The second domain, named CR3 and spanning amino acids 38 to 98, contains a zinc-binding domain. This domain shows partial homology to the primary structure of the two zinc-binding domains of the HPV E6 protein, however, these form divergent secondary and tertiary structures (Berezutskaya et al., 1997).

3.2.1.2 Disruption of cell cycle control and episomal maintenance

E7 protein primarily targets and degrades pRb and the related p107 and p103 proteins. The interaction is mediated through the LXCXE motif contained within the CR2h domain of

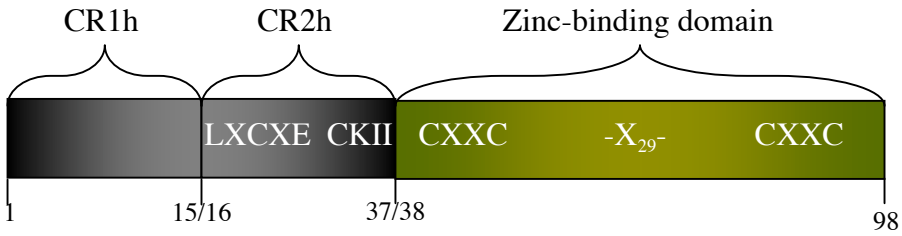


Figure 15.
Schematic representation of the E7 protein.
The amino-terminal domain shows sequence homology to a region of CR1 and CR2 of adenovirus protein E1A (CR1h and CR2h). Within the CR2h region is the pRb binding site (LXCXE) and the casein kinase II phosphorylation site (CKII). The C-terminal zinc-binding domain mediates E7 interaction with several cellular partners.
Adapted from Munger *et al*. 2004.

E7 and is aided by the CR1h domain (Fig. 15). These domains also mediate pRb interaction in both the adenovirus E1A and Simian Virus 40 (SV40) T antigen proteins (Longworth and Laimins, 2004b). Generally, high-risk HPV E7 proteins bind pRb with higher affinity than low-risk HPV E7 proteins and mutations in the LXCXE adversely affect this interaction which is required for immortalization (Flores et al., 2000). Additionally, high-risk HPV E7 proteins are able to bind and neutralise the CDK inhibitors p21 and p27 and stabilise CDK/cyclin complexes and cyclins (Jones et al., 1997; Zerfass-Thome et al., 1996).

E7 induces 26S proteasome-mediated proteolysis of pRb in a system that is not completely understood. However, it has been documented that both pRb-binding N-terminal domain and the non-binding C-terminal domains of E7 are required for degradation of pRb (Oh et al., 2004a) and to override p21-mediated growth arrest (Jones et al., 1997). Additionally, E7 protein from the HPV 1, a low-risk HPV that is able to bind with high affinity to pRb is unable to mediate its degradation. This strongly suggests that other proteins are required to interact with E7 for pRb degradation (Berezutskaya et al., 1997; Longworth and Laimins, 2004b). All these functions contribute to the principle effect of E7 that is to decrease E2F negative control leading to G_1 to S–phase progression. E2F also positively regulates mitotic control genes and therefore a consequence of E7 deregulation is a positive effect on the G_2 to M-phase checkpoint (Thierry et al., 2004). E7 also binds Histone Deacetylases (HDACs) via its C-terminal zinc-binding domain for which the integrity of the fold and the original amino acid at position 67 has been demonstrated to be critical (Brehm et al., 1999). HDACs repress transcription of genomic promoters through deacetylation of core histone proteins and in so doing remodel chromatin. E7 repression of HDACs has been associated with the maintenance of viral episomal DNA in HPV-infected cells (Longworth and Laimins, 2004a). Therefore, E7 reduces negative regulation and increases positive regulation of the cell cycle.

3.2.1.3 E7 and genomic instability

The principle contribution of E7 to genomic instability associated with high-risk HPVs is at the centrosomes. Centrosomes are responsible for correct chromosome segregation during mitosis and abnormal multipolar mitoses in suprabasal epithelial cells is regarded as a mark of high-risk HPV lesions (Duensing and Munger, 2004). E7-induced errors are obtained by uncoupling of centrosome duplication from the cell cycle (Duensing et al., 2000) is mediated in both a Rb-dependant and Rb-independent manner. Indeed, deletion of Rb in transgenic mice has revealed a Rb-controlled mechanism for centrosome synthesis (Balsitis et al., 2003) whereas the negative regulation of p21 and p27 by E7 has been identified as being implicated in the non-Rb mediated pathway (Duensing and Munger, 2004).

3.2.1.4 E7 degradation

E7 is itself degraded by the Ub-26S proteasome system. Poly-Ub E7 has been documented (Wang et al., 2001) and degradation of E7 in this way has been uncoupled from E7-mediated degradation of pRb (Gonzalez et al., 2001). E7 has been reported to interact with the S4 subunit of the 26S proteasome which could suggest non-Ub mediated degradation, however, it has been demonstrated that this is not required to promote degradation of pRb and its associated proteins. Indeed, mutation of the amino acid at position 91, necessary for the zinc-binding fold, prohibits S4-binding yet these mutants remain competent in pRb degradation (Duensing and Munger, 2003).

Degradation of E7 has been found to be performed in the nucleus of CaSki cells and mediated by the SCF complex (Oh et al., 2004a). It was found that the E2 Ub-conjugating enzyme involved in the ubiquitination was the UbcH7 protein. Additionally, it was reported that in the same study that although the half-life of E7 was increased in Mouse Embryo Fibroblasts (MEFs) deficient in a component of the SCF complex (Skp2), partial E7 degradation persisted (Oh et al., 2004a). This may be explained by a second pathway for Ub-26S proteasome degradation of E7 protein which is mediated by Suppresser Of Cytokine Signaling-1 (SOCS1). SOCS1 has been reported to interact with the E7 protein in the nucleus of HeLa cells and provokes its degradation in the cytosol (Kamio et al., 2004). This property of SOCS1 is associated with its interaction with the components of the VCB-type E3 Ub-ligase complex where it acts as an adapter molecule (Kamio et al., 2004).

3.2.2 E6

3.2.2.1 The E6 protein

E6 is a small, basic zinc-binding protein. E6 from HPV16 can use one of two methionines as a start codon giving rise to a protein of either 151 or 158 amino acids (Fig. 16). The conserved cysteines at positions 32, 35, 65 and 68 along with those at positions 105, 108, 138 and 141 co-ordinate Zn^{2+}, forming structurally analogous but functionally divergent zinc-binding domains (Dr Gilles Travé UMR 7100, personal communication). "Natural" proteolysis of purified E6 samples degrade into two soluble entities which have been termed Zinc Domain (ZD) 1 and 2 (Nomine et al., 2003) (Fig. 16). In the cell, E6 protein is involved in both protein:protein and protein:DNA interactions which are described in the proceeding section. Point mutation analysis has allowed the definition of E6 domains or regions implicated in these interactions, schematised in Fig. 16.

3.2.2.2 Cellular partners of E6 protein

There are a number of E6 protein partners described in the literature over the past 15 years. However, the proceeding description of the E6 binding partners does not follow an order with respect to historical importance as a majority of these can now be arranged into groups with respect with their E6-binding characteristics.

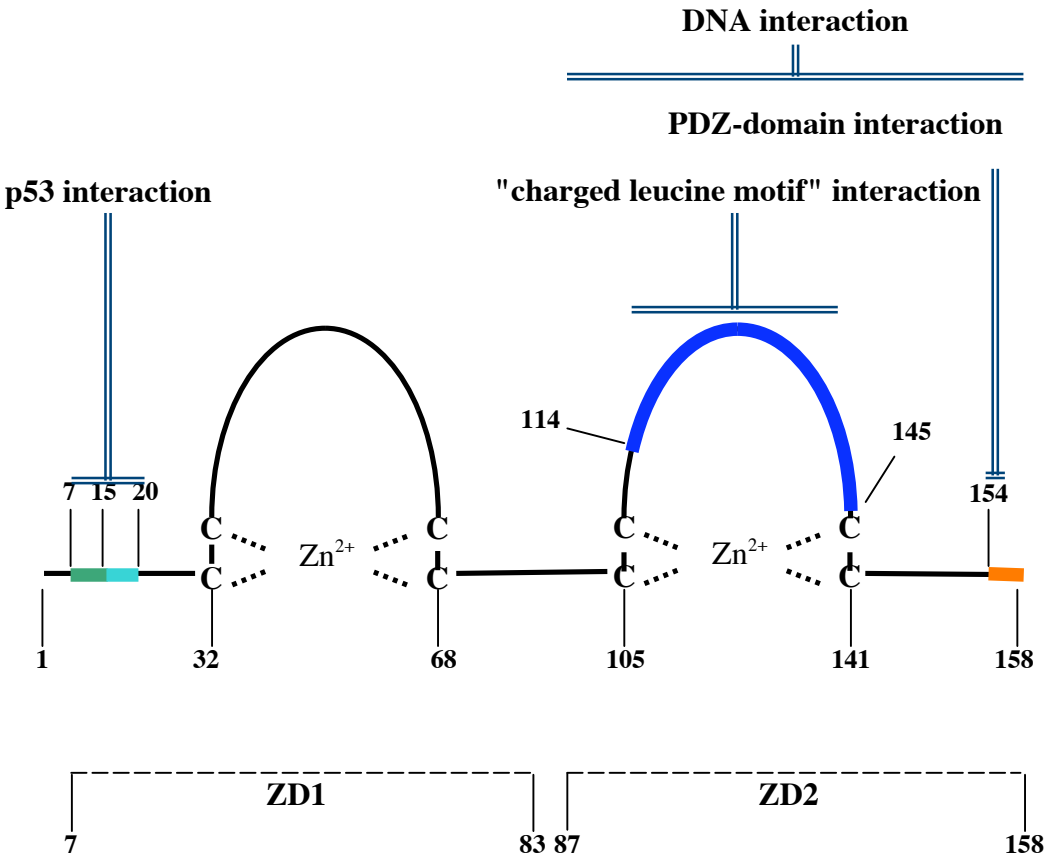


Figure 16.
Schematic representation of 16E6 protein.
16E6 has two methionines that can be used as start codon giving rise to a protein of either 151 or 158 amino acids. The later form is represented here with annotations indicating interacting domains obtained from mutational analyses. The two zinc-binding domains (ZD) are structurally disassociated by proteolysis. The resulting polypeptides are termed ZD1 and ZD2.
Adapted from Ristriani et al. 2000, Nominé et al. 2003, Thomas and Banks 2001, Cooper et al. 2003, and Liu et al. 1999.

3.2.2.2.1 Proteins interacting through a "charged leucine motif"

In 1998 it emerged that E6 interacted not only with E6-AP via a "charged leucine motif" but also with many of its known cellular partners. The consensus motif was found to be LXXLL(A/G)X which was obtained from independent experiments. These results were the first to group E6-Associated Protein (E6-AP), Paxillin and E6-Binding Protein (E6-BP) also named ERC-55 as binding E6 through the same motif (Chen et al., 1998; Elston et al., 1998; Vande Pol et al., 1998). Since then, the importance of this motif has been extended to other protein partners of E6 and curiously also other cellular partners of E6-AP.

3.2.2.2.1.a E6-AP

E6-AP (E6-Associated Protein) is a 100KDa protein which was initially found by association to p53 and later implicated in Angelman syndrome (Nawaz et al., 1999). It has been found to be an E3 Ub-ligase, the first of the HECT domain proteins (Huibregtse et al., 1995). Alone it does not bind to p53 but in the presence of E6 it forms a trimeric complex that results in 26S proteasome-mediated degradation of p53 (Cooper et al., 2003). The tertiary structure of E6-AP has yet to be defined, however, the binding site for E6 interaction has been mapped to the region spanning the amino acids 391-408. This segment has been demonstrated to be necessary and sufficient for E6 binding (Huibregtse et al., 1993) and has been structurally characterised (Be et al., 2001). This 18 amino acid peptide (e6ap) demonstrates an α-helical structure in solution. The leucine residues of the consensus motif LXXLL at positions 9, 12 and 13 are presented on the same side of the helix and there is a sterical requirement of Glycine or Alanine at position 14 (Be et al., 2001). The importance of these amino acids was also demonstrated in this study by mutational analysis (Be et al., 2001) which has been independently confirmed (Bohl et al., 2000). It has been found that both low- and high-risk E6 may alone bind to a C-terminal region of p53 spanning or adjoining the amino acids 376 to 384. However, E6-AP promotes E6 binding to the p53 "core" domain (amino acids 101 to 303) of only high-risk E6 which has been demonstrated to be required to induce the degradation of p53 (Li and Coffino, 1996a). It is not clear whether this "modulation" of E6 binding to p53 is a property of an E6/E6-AP complex or whether E6-AP acts on an E6/p53 complex. Mutation of the C-terminal Lysines at positions 370, 372, 373, 381, 382 and 386 of p53 which are targeted for ubiquitination results in a p53 protein

(p536KR) which retains transcriptional activity yet is resistant to MDM2-mediated degradation (Rodriguez et al., 2000). However, in this study, the ubiquitination of p53 protein by MDM2 was not studied (Rodriguez et al., 2000). Camus *et al.* report that, although these six lysines are favourably targeted by MDM2 for ubiquitination, other lysines may also be ubiquitinated by MDM2 when present in excess. These conditions are probably not representative of physiological conditions but demonstrate the existence of alternative lysines open to ubiquitination. Furthermore, the same authors communicate that there is a marked difference in the ubiquitation of p53 when mediated by MDM2 or E6 (Camus et al., 2003). MDM2 was seen to produce a ladder pattern of ubiquitination representing multiple monoubiquitination of p53. E6, on the other hand, did not induce such a ladder pattern yet efficiently degrades both wild type p53 and the p536KR mutant proteins. However, E6-mediated degradation of p53 has been shown to require ubiquitination (Asher et al., 2002) suggesting that MDM2 and E6 promote the ubiquitination of different lysines and possibly following different mechanisms. Camus *et al.* propose that E6 may induce the addition of poly-ubiquitin chains to p53 creating extremely high molecular weight species as opposed to the ladder pattern familiar to MDM2-mediated ubiquitination (Camus et al., 2003). The difference in ubiquitination pattern may also be explained by the E4 Ub-ligase activity demonstrated by p300/CBP which is required for poly-ubiquitination and efficient degradation of p53 by MDM2 (Berger et al., 2001; Grossman et al., 2003; Zhu et al., 2001). Indeed, MDM2 and E6-AP are representative of the HECT domain and the RING finger domain E3 Ub-ligases respectively. These two classes differ in the approach to substrate ubiquitination (Fig. 7) which could be fundamental in understanding the different results obtained with E6 and MDM2. The questions arising from the reported divergence in MDM2- and E6-mediated p53 ubiquitination remain unanswered.

Historically, the "charged leucine motif" was originally identified on transcription factor co-activators that bind to steroid hormone receptors in the presence of their ligand which is required for expression of hormone-specific genes (Bohl et al., 2000; Savkur and Burris, 2004). E6-AP, along with another HECT domain protein (hRPF1), have recently been found to have steroid hormone receptor co-activation function (Henderson et al., 2002; Nawaz et al., 1999) and to simultaneously mediate degradation of these in a 26S proteasome-dependant fashion (Moraitis and Giguere, 2003). The primary function of transcriptional co-activators is to act as a bridge between DNA-binding transcription factors and the basal transcriptional machinery. This additional role of E6-AP in transcriptional co-activation may

be related to the Angelman Syndrome in which mutations occur in E6-AP outside the catalytic domain for Ub-ligase activity (Cooper et al., 2004).

3.2.2.2.1.b E6BP

E6 Binding Protein (E6BP), also known as ERC-55, is a calcium binding protein and part of the CREC family of proteins (Honore and Vorum, 2000). It has been found that cervical carcinoma keratinocytes are resistant to stimuli that induce terminal differentiation such as serum and calcium. This resistance has been found to be, at least in part, due to E6 interaction with E6BP (Sherman et al., 2002). Chen *et al*. isolated the minimal binding domain of E6BP as being LEEFLGD by mutational analysis. This was independently confirmed by Elston *et al*. who used yeast-two-hybrid technology to screen a peptide library for E6-binding partners and aligned peptides with a consensus motif E/D L L/V G with the amino acid sequences of both E6BP and E6-AP (Chen et al., 1998; Elston et al., 1998).

3.2.2.2.1.c MCM7

MultiCopy Maintenance Protein 7 (MCM7) (Kuhne and Banks, 1998), also known as MiniChromosome Maintenance 7 (Kukimoto et al., 1998), is a protein which is essential for DNA replication and ensures one round of DNA replication per cell cycle (Kukimoto et al., 1998). E6 has been found to cause the degradation of MCM7 in the presence of E6-AP. Both E6 and E6-AP have been found to interact with MCM7 through its LLG motif and E6-AP has been demonstrated to cause the degradation of MCM7 in the absence of E6 (Kuhne and Banks, 1998).

3.2.2.2.1.d Paxillin

Paxillin forms part of the focal adhesion protein complex, which adhere to the extracellular matrix and are essential in cellular morphology, division and metastasis (Tong et al., 1997). E6 protein has been demonstrated to interact with paxillin, preventing its association with its cellular partners such as vincullin and the focal adhesion kinase (Tong et al., 1997). E6 binds the consensus motif LDXLLXL (highly conserved amino acids are

underlined). This sequence can be found four times in the sequence of paxillin (Vande Pol et al., 1998) and the most important for E6 binding appears to be the most N-terminal of the four, the LD1 motif ^{3}DLDALLADLESTT^{15} (Tong et al., 1997). Additionally, Zyxin, another focal adhesion protein, has also been found to interact with E6 but only from HPV6 (Degenhardt and Silverstein, 2001). Although both E6 and E6-AP both bind the same motifs in paxillin, it is not clear whether these collaborate to induce degradation of paxillin as "simple" E6-paxillin interaction has been demonstrated to affect its cellular function (Vande Pol et al., 1998).

3.2.2.2.1.e TNF R1

Tumour Necrosis Factor (TNF) is a pro-apoptotic signalling molecule which induces intracellular apoptotic signals through its cell surface receptor, TNF R1. E6 has been found to bind to the C-terminal region of this receptor spanning amino acids 412 to 435 which include two E/DLL/VG motifs isolated by Elston *et al.* as discussed earlier (^{412}PRREATLELLGRVRDMDLLGCL^{435}) (Elston et al., 1998; Filippova et al., 2002). E6 binding was found to interfere with TNF R1-associated death domain (TRADD) binding to TNF R1 which is necessary for intracellular signalling.

It is interesting to note that many of the proteins described above interact with E6 and E6-AP using a single domain. The exact nature of the interplay between binding E6 and/or E6-AP is still not completely understood, however, interaction of E6 with its cellular partners through this domain has been found to be highly important for its cellular functions. Indeed, mutational analysis of 16E6 demonstrates that E6 mutant proteins not able to bind the "charged leucine motif" partners (E6-AP and BP were studied here) were less capable of immortalizing cells (Liu et al., 1999) and demonstrated reduced oncogenic potential (Nguyen et al., 2002). Another mutational study was performed using Bovine Papillomavirus type 1 E6 (BE6). This protein is not capable of inducing the degradation of p53, yet interacts with E6-AP, E6BP, paxillin and immortalizes cells. It was found that a BE6 mutant not capable of interacting with E6-AP, E6BP and paxillin was incapable of inducing immortalization of cells (Das et al., 2000). However, in this study, the BE6 mutants that lost their affinity for E6-AP and E6BP retained substantial paxillin-binding activities and were able to immortalize cells.

This observation may be explained by the multiple "charged leucine motifs" of Paxillin which may contribute to more effective binding to E6 through additional contacts. Furthermore, the e6-ap peptide has been demonstrated to inhibit the interaction of E6-AP with E6 (Liu et al., 2004).

3.2.2.2.2. PDZ domain proteins

PDZ (**P**SD-95/**D**lg/**Z**O-1) domain proteins play a role in the regulation of the cell cycle, differentiation and cellular morphology (Nguyen et al., 2003). These have been found to be important at the cellular membranes acting as scaffold proteins, organising structural and signalling protein complexes (Watson et al., 2003). High-risk and not low-risk HPVE6 proteins contain the C-terminal PDZ-domain binding motif S/T X V/L which requires a free carboxy terminus. High-risk E6 targets the membrane-associated guanylate kinase (MAGUK) family proteins: Drosophila discs large-1 (hDlg), MAGI-1, -2 and –3, along with the leucine-rich repeat and PDZ domain (LAP) family member, Scribble (Watson et al., 2003). Degradation of hDlg and MAGIs has been reported to be independent of E6-AP, yet these are targeted by E6 for degradation, mediated by ubiquitination and the 26S proteasome (Sterlinko Grm et al., 2004). This indicates that, for these proteins, other E3 Ub-ligases must be recruited by E6.

Another PDZ protein that interacts with E6 is E6-Targeted Protein 1 (E6TP1), a Rap GTPase-activating protein or Rap GAP. Rap GAP proteins reduce the levels of GTP-bound Rap activated protein. Activated Rap stimulates the mitogen-activated protein kinase following growth factor binding to cellular receptors and constitutively activated Rap can transform fibroblasts (Gao et al., 2001; Singh et al., 2003). It has been shown that both E6/E6-AP and E6-AP alone induce degradation of E6TP1 via the ubiquitination-26S proteasome system (Gao et al., 2002). It is interesting to note that although E6TP1 has a PDZ domain, Gao *et al*. isolated the 194 amino acids composing the C-terminus of E6TP1 as being the minimal binding site for E6 (Gao et al., 1999). The same group refined this to 42 amino acids spanning the region 1716-1758 that constitutes a leucine zipper motif (Gao et al., 2002). This motif ($^{1716}LX_6LX_6LX_6EX_6LX_6L^{1758}$) is much more extended than the "charged leucine motif" discussed above, however, this does not preclude the existence of a cryptic "charged leucine motif" within this stretch. Interestingly E6 also targets homologous Rap GAP, Tuberin that is implicated in insulin signalling (Lu et al., 2004).

3.2.2.2.3 Other proteins

The following proteins have been described as cellular binding partners of the E6 protein but, as yet, cannot be classified as having "charged leucine motifs" or PDZ domains.

3.2.2.2.3.a p300/CBP

p300 and the CBP have overlapping functions and demonstrate 63% homology. They are associated with important cellular processes such as cell cycle regulation, proliferation, apoptosis, differentiation and DNA-damage response (Iyer et al., 2004). Their principal function is to act as transcriptional co-activators, as described previously for E6-AP, linking transcription factors to the basal machinery. Additionally, these two proteins demonstrate acetyltransferases activity towards various substrates. Acetylation of histones is a well-documented function of these proteins and is associated with highly transcriptionaly active genes. p53 is also a substrate for p300/CBP mediated acetylation, stabilising and activating p53 protein in response to signalling pathways (Grossman, 2001). Only high-risk HPVE6 proteins are capable of binding p300/CBP and this is mediated by a minimal domain that is common to both p300 and CBP that is the amino acid sequence GCKRKTNGGCPVCKQLIAL. In p300, this region spans the amino acids 1770 to 1788 and in CBP, it spans the amino acids 1770 to 1788 (Zimmermann et al., 1999). Interaction of E6 with p300/CBP does not result in degradation but prohibits p300/CBP-p53 interaction, down regulating p53 transcriptional activity (Zimmermann et al., 1999). Although E6 demonstrates both transcriptional activation and repressive activity, the E6-p300/CBP complex does not have transcriptional activating function (Zimmermann et al., 2000). An interesting aside is the report made by Hengstermann *et al*. who describe a "complete switch from MDM2 to HPV E6-mediated degradation of p53 in cervical cancer cells" (Hengstermann et al., 2001). This could be due to functional inactivation of p300/CBP by E6, prohibiting the poly-ubiquitination and thus degradation of p53 by MDM2. Interestingly, in SiHa cells, the sequence encoding the amino acids 940 to 1167 of p300 has been found to be deleted (Iyer et al., 2004). However, it is not clear whether this abrogated p300 has any functional effect or whether CBP could replace p300 in overlapping roles.

3.2.2.2.3.b Tyk and IRF-3

A group of structurally related transcription factors has been implicated in mediation of cellular response to interferon. This group is called either the Interferon α-Stimulated Gene Factor (ISGF) or Interferon Regulatory Factor (IRF). Interferon Regulatory Factor-3 (IRF-3) is a transcription factor critical in the regulated-expression of the IFNα and β controlled genes. E6 of high-risk HPVs binds but does not induce the degradation of IRF-3 and, in doing so, may play a role in disrupting the cellular anti-viral response (Ronco et al., 1998). The E6-binding site on IRF-3 has not been determined, but it is proposed to be contained within the region of ^{138}DILDELLGNMV^{148} which would place it among the "charged leucine motif" family (Be et al., 2001). Another protein, ISGF3α, homologous to IRF-3, is activated by phosphorylation by TyK2, a tyrosine kinase. High-risk HPVE6 proteins associate with TyK2 and impair its activity, deregulating another part of the interferon signalling cascade (Li et al., 1999). The interaction domain of E6 with these two proteins has yet to be elucidated.

3.2.2.2.3.c Bak

Bak is a pro-apoptotic member of the Bcl-2 family and is targeted for degradation by E6/E6-AP. Bak binds directly to E6 of both high- and low-risk HPVs although more strongly to high risk HPVE6. E6-mediated ubiquitination and degradation occurs through a trimeric complex of E6/E6-AP/Bak (Thomas and Banks, 1999) and is regulated in normal cells by E6-AP mediated ubiquitination and degradation (Jackson et al., 2000). However, the E6 binding site on Bak has yet to be determined.

3.2.2.2.3.d E6 binding to myc, expression of hTERT and telomerase activity

Normal somatic cells are able to undergo a finite number of divisions. A major factor limiting this number of cellular divisions is telomere shortening. Telomeres are specialised chromosome structures consisting of repetitive DNA sequences and specialised proteins. At each cellular division the duplication of genomic DNA a phenomenon inherent to replication of linear DNA causes a reduction of chromosome termini, which is often equated to cellular ageing at a molecular level. Indeed, critically short telomeres induce senescence and may

even cause apoptosis. Senescence is a tumour suppressive function, precluding immortalization. Immortalized cancer cells maintain telomere length by activating telomerase, an enzyme that synthesises telomeric repeat DNA (Horikawa and Barrett, 2003). A key catalytic protein subunit of human telomerase is the telomerase reverse transcriptase (hTERT). E6 interacts with the transcription factor protein Myc and induces hTERT gene expression. It is not clear however, whether this involves the co-activating properties of either E6-AP or p300/CBP (Veldman et al., 2003). Furthermore, E6 induces the degradation of a telomerase promoter suppressor (Gewin et al., 2004).

E6 also shows transcriptional activation and repression of other genes, for example: the Major Early promoter of the HPV genome, described earlier; Fibronectin, an cellular-adhesion protein (Shino et al., 1997); TGF-β1, a regulator of cell growth and differentiation (Dey et al., 1997) and c-fos, a proto-oncogene implicated in cell cycle regulation (Morosov et al., 1994). However, the exact nature of this transcriptional control is not well understood and may be an indirect effect resulting in E6-mediated p53 degradation. Additionally, E6 also binds four-way DNA junctions (Ristriani et al., 2000; Ristriani et al., 2001), however, the physiological significance of this function has yet to be determined.

3.2.2.3 Degradation of E6

It has been observed that proteasome inhibition increases the steady-state level of high-risk HPVE6 but not of low-risk HPVE6 and that this was independent of E6-AP binding and of E6-mediated degradation of p53 (Kehmeier et al., 2002). A supplementary study demonstrated that although both low- and high-risk HPVE6 proteins are subjected to ubiquitination and 26S proteasome degradation (Stewart et al., 2004). In the later study, a difference in the ubiquitination profile was noted between high- and low-risk HPVE6. An intriguing difference in the two approaches used for these studies is that the first study (Kehmeier et al., 2002) used an epitope tag fused to the C-terminus of E6 whereas the later (Stewart et al., 2004) used an N-terminal tag. The former construction, as Stewart *et al.* note, precludes E6 interaction with PDZ-domain proteins that may explain the contradicting observations made for low-risk E6 degradation. This may be linked to the report that E6-AP is not implicated in E6-mediated degradation of PDZ-domain proteins, implicating that other E3 Ub-ligases are capable of interacting with E6. However, it is unlikely that degradation of E6

involves PDZ-domain containing proteins, at least for high-risk types, as the E6 C-terminal fusion protein used by Kehmeier *et al.*, which prohibit PDZ domain interaction, were effectively degraded.

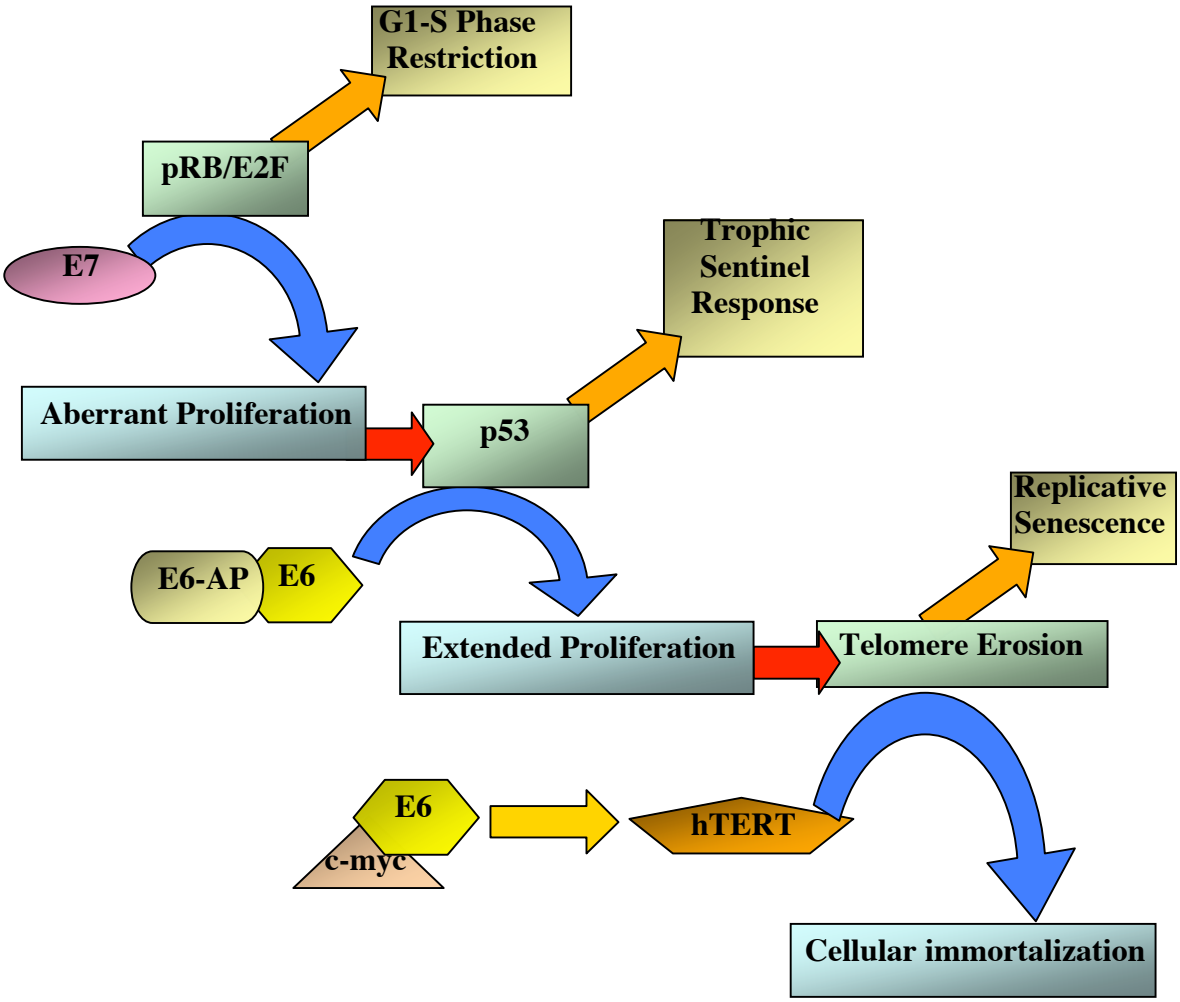


Figure 17.
Critical steps in E6/E7 immortalization.
The inactivation of pRb, p53 and telomere erosion pathways are required for cellular immortalization mediated by E6 and E7.
Adapted from Munger et al. 2004.

3.3 E6 and E7-mediated cellular immortalization: conclusion

The critical steps in E6/E7 mediated immortalization have been thought to be summarised by first E7-mediated proliferation, E6-mediated degradation of p53 and telomere maintenance (Fig. 17). However, the myriad of accompanying functions allocated to E6 and E7 may play an equally important role in this. HPV-transformed cancer cells therefore present complex intra- and extra-cellular modifications that result from integration and expression of E6 and E7 genes.

4. Introduction of the research projects

Cellular transformation induced by oncogenic high-risk HPVs is directly attributed to the cellular effects of the E6 and E7 oncoproteins. These proteins interact with cellular factors to induce transformation. Characterisation of these interactions is therefore of greatest importance for the understanding of tumourigenesis. Furthermore, the process of transformation mediated by E6 and E7 oncoproteins produces distinct cellular modifications which can be exploited for the development of therapeutic strategies.

In our laboratory, work has been performed to characterise the structure-function of 16E6 and to develop therapeutic molecules for the treatment of cervical cancer. The work presented here deals with both these areas of research. We used phage display to isolate peptides that target and internalise into cervical cancer cells. These peptides are the leads for targeted therapeutic strategies such as the re-targeting of diphtheria toxin. In a second series of experiments, we used phage display to isolated peptide ligands for 16E6 protein. These allowed us to identify domains in p53 protein important for its degradation.

Before presenting the work performed, I will first describe the phage display technology.

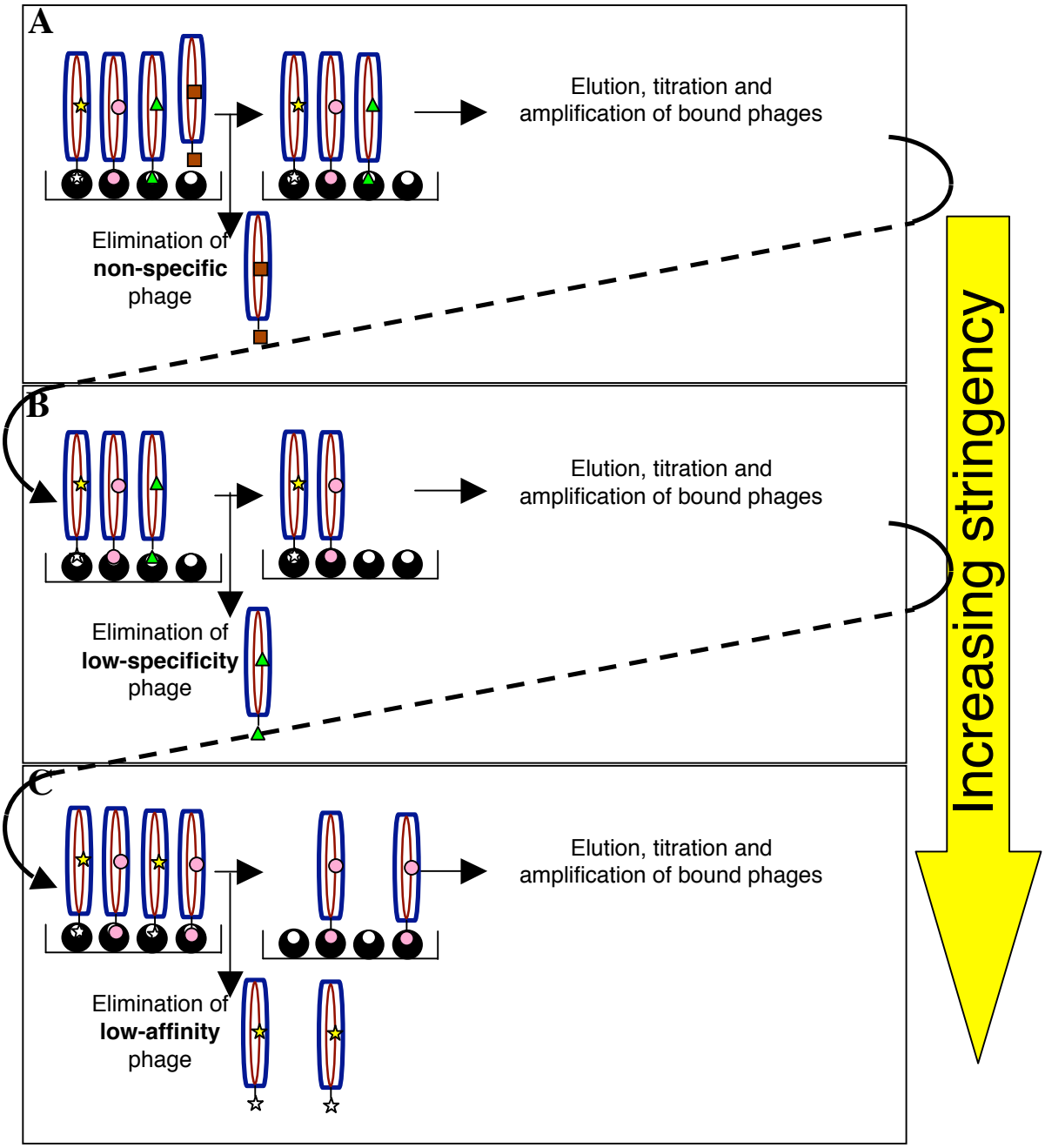


Figure 18.
Principle of phage display.
The process of affinity selection resides on successive rounds of biopanning on immobilised substrate. Each round of biopanning consists of selection of binding phage particles and their amplification in *E. coli*. The amplified output will be enriched with binding particles. Increasing stringency in subsequent rounds of selection with augments affinity selection pressure, thus enriching the isolated sub-population in high-affinity binding particles. After 3 or 4 rounds of biopanning, individual clones of selected phage are analysed by sequencing their genetic material from which is deduced the amino acid sequence of the protein or peptide presented at their surface.

5. Phage display

5.1 Introduction

George Smith first described the principle of the phage display technology in 1985 (Smith, 1985) and the affinity selection of phage particles displaying specific epitopes from a mixture of phage particles (biopanning) in 1988 (Parmley and Smith, 1988). This technology is based on the observation that polypeptides, fused to bacteriophage coat proteins, may be displayed on the phage particles that encapsidate the encoding gene. This physical association between the phage genotype and phenotype allows rapid determination of the displayed molecule through DNA sequencing and lends itself to the screening of diverse combinatorial libraries of peptides and proteins. Indeed, this technology was first used for epitope mapping of antibodies using combinatorial peptide libraries (Scott and Smith, 1990) and the affinity selection of combinatorial recombinant antibody fragment libraries comprised of antigen-binding fragments (Fabs) or single-chain variable region fragments (scFvs) (Huse et al., 1989; McCafferty et al., 1990).

5.2 Biopanning and M13 phage

5.2.1 Biopanning, the principle of phage display selection

The principle defining the process of biopanning is the selective enrichment of individual phage clones by successive rounds of retention and amplification of the phage display library. Although innovative biopanning methodologies have since been described and will be later discussed, Parmely and Smith (Parmley and Smith, 1988) initially described a method which is still applicable today (Fig. 18). They incubated displaying phage particles with antibodies immobilised on plastic and, whereas non-bound particles were washed away, binding clones were eluted with low pH solution and/or amplified by bacterial infection. This constitutes one round of selection, three or four rounds of biopanning are usually performed. After these successive rounds of retention and amplification, the final phage population is enriched with binding clones and the amino acid content of the displayed peptides or polypeptides is deduced from the DNA encoding it. Biopanning is a rapid and efficient process that needs no special equipment. The diverse nature of the molecules capable of being

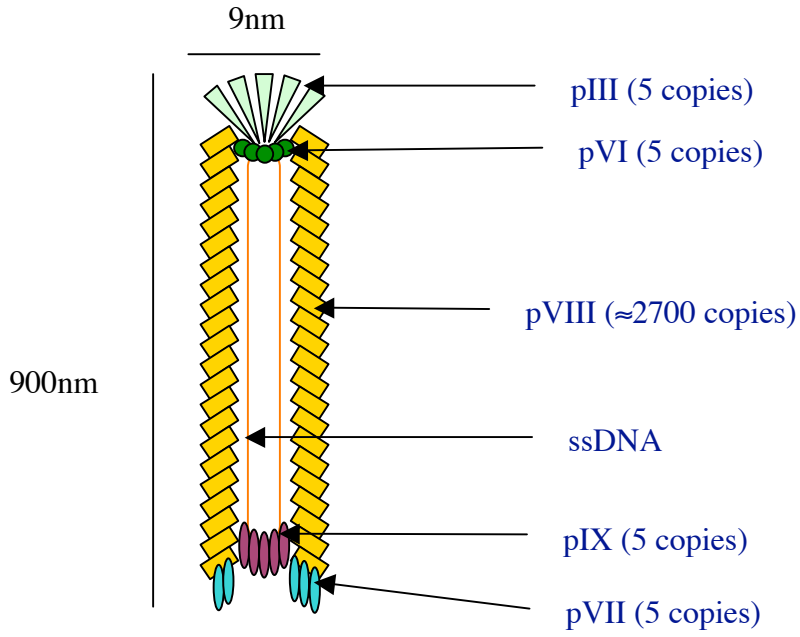


Figure 19.
Schematic representation of M13 phage.
Wt M13 phage consists of a ssDNA genome (6.4 Kb) surrounded by approximately 2700 copies of the major coat protein pVIII and capped at one end by 5 copies of both pIX and pVII and at the other end by 5 copies of pIII and pVI.

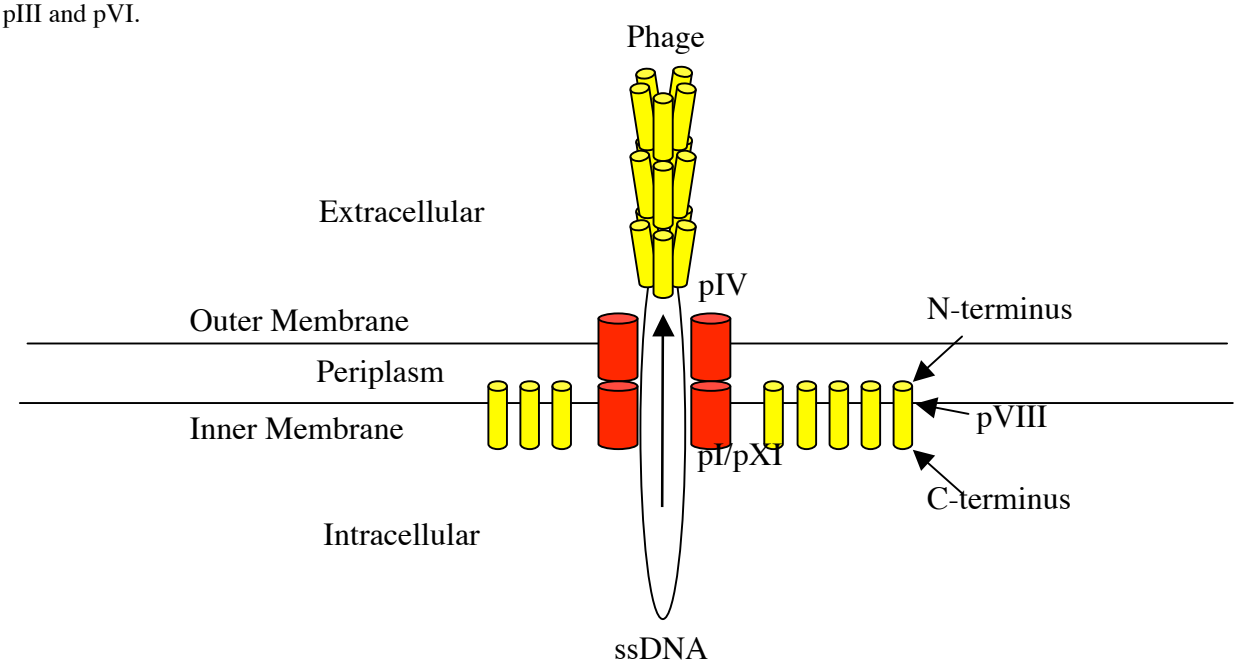


Figure 20.
Assembly of M13 bacteriophage by extrusion through bacterial membrane.
Coat proteins assemble in the bacterial innermembrane, their N-termini in the periplasm. For clarity, minor coat proteins initiating (pVII and pIX) and terminating (pIII and pVI) phage assembly are omitted. The phage genome, composed of single-stranded DNA, is extruded from the host at assembly sites. These are composed of phage-encoded proteins (pIV, pI and pXI) that span both the inner and outer membranes of the host cell. At these assembly sites, coat proteins interact with the ssDNA and are thus transferred from the inner bacterial membrane to the assembling phage particle that occurs without cellular lysis.
Roth *et al*. 2002.

presented on filamentous phage and the robust nature of the phage particle allows great flexibility in terms of experimental design and has contributed to its application in a wide range of areas.

5.2.2 M13 phage display vectors

The great majority of phage display libraries use the structurally homologous Ff family of filamentous phage, particularly M13 (Colas, 2000; Rodi and Makowski, 1999), although other types of phage have been used as display vehicles, for example T7 and lambda (Willats, 2002). Wild type M13 (Fig. 19) are 900nm in length, 9nm in diameter and comprise of a ssDNA surrounded by a protein coat. Infection of bacteria is of temperate nature (non-lytic) and requires interaction between the pIII coat protein and the F pillus of “male” bacteria. Inside the cell, the ssDNA is converted to dsDNA and used as the template for the synthesis of both phage proteins and ssDNA. A phage-encoded pore complex and the phage coat proteins localise in the bacterial inner membrane, the N-termini in the periplasm and C-termini in the cytoplasm (Fig. 20). Assembly of phage particles by extrusion through this pore complex is initiated by pVII and pIX, continued by pVIII and terminated by pIII and pVI (Roth et al., 2002).

Presentation of peptides or proteins at the surface of M13 phage is obtained by the genetic fusion of the sequence coding for a coat protein to that coding for the amino acid sequence to be displayed. The hybrid gene is then inserted into either the phage genome or a phagemid expression vector. These two approaches may be used to produce phage particles that present on their surface either multiple or single copies of the peptide. In the case of systems based on phage genomes, all the required proteins for viable phage particle assembly with the addition of the hybrid gene are encoded by the genome and are expressed in infected bacteria. The resulting phage particles are assembled from these proteins giving rise to phage particles which include the protein product of the hybrid gene encoding a capsid protein. Therefore, these present at their surface, fused to the capsid protein in question, the desired amino acids. In the case of phagemid vectors, however, only the hybrid gene is expressed in bacteria. The other proteins required for phage particle assembly are supplied by "helper

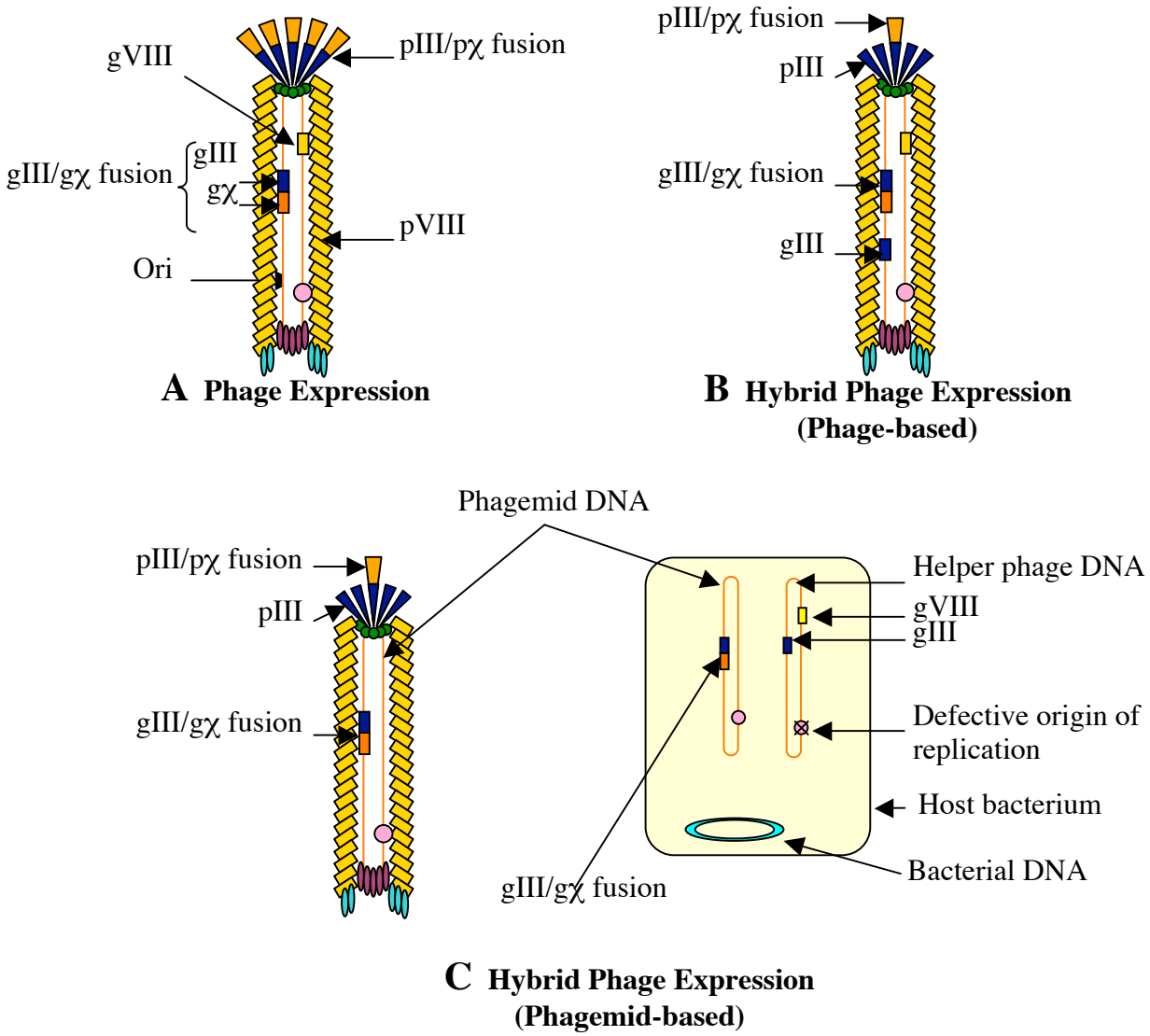


Figure 21. Phage display formats.
(A) The simplest format for phage display is to fuse the gene (gχ) encoding a foreign protein (pχ) to one of the coat proteins. In this example, pIII was chosen although fusion to other coat proteins is also possible – see text for details. This produces phage particles in which all copies of the given coat protein are fusion proteins (pIII/pχ). (B) Hybrid phage can be made by incorporating a second, native, copy of the fusion coat protein. This gives rise to phage particles with a mix of fusion and native coat protein. (C) Phagemid-based systems utilise genes encoded by both the phagemid and helper phage for the construction of viral particles.
Adapted from Willats 2002.

phage" infection. These are M13 phage containing a mutation in their genome which prohibits replication and thus allows preferential phagemid encapsidation.

Although all five coat proteins have been used for M13 phage display (Sidhu, 2001), the great majority of systems use either the minor coat protein, pIII, present at 5 copies per particle or the major coat protein, pVIII, present, on wild type M13, at 2700 copies per particle. For phage based systems (as opposed to phagemid) the hybrid gene may replace the wild type gene encoding the coat protein in which case all copies of the coat protein in the resulting phage particle will be display the amino acid sequence. However, where both the wild-type and hybrid gene are present on the phage genome then these two proteins will compete for inclusion into phage particles. Resulting particles will therefore present the amino acid sequence only on a certain proportion of the capsid proteins. Conversely, phagemid based systems use the proteins of the helper phage for particle assembly and therefore will inherently produce hybrid particles. However, helper phage, deficient in the appropriate coat protein gene allow 100% presentation with phagemid based systems (Fig. 21) (Willats, 2002). An interesting point to note here is that presentation on the surface of phage particles can only be theoretically quantified as proteolysis may significantly reduce the "effective" number of presented sequences (Fernandez-Gacio et al., 2003).

Thus, using the pIII it is theoretically possible to present of up to 5 copies of an amino acid sequence per phage particle and using pVIII, up to 2700. This is important where the avidity of binding interactions is taken into account. However, structural considerations limit the free use of either of these systems. Indeed, the nature of wild type pVIII protein (Sidhu, 2001) limits presentation of peptides composed of more than 10 amino acids (Iannolo et al., 1995) confining presentation of polypeptides to the pIII coat protein. Furthermore, presentation has been restricted to N-terminal display using either pIII or pVIII as their C-termini interact with other phage coat proteins and the encapsidated DNA respectively (Sidhu, 2001). This has prevented the use of phage display in certain applications, for example where a free C-terminus is required for the determination of PDZ domain-binding ligands (Sidhu et al., 2003). More recently, Sachdev Sidhu's group have used protein engineering of address pIII and pVIII C-terminal presentation (Fuh and Sidhu, 2000; Weiss and Sidhu, 2000) and pVIII-polypeptide presentation (Weiss et al., 2000).

5.3 Applications of phage display

5.3.1 Protein display

5.3.1.1 Antibodies

Immunisation of mice and the subsequent immortalization of secreting B cells by fusion with a myeloma cell line to form hybridomas has provided a wide repertoire of murine antibodies with desired specificity for a given antigen. The immunogenicity of murine antibodies has somewhat impeded their utility in human therapeutic applications. Production of human antibodies is restricted by the limited range of antigens, by technical difficulties and for ethical reasons. Methods have sought to "humanise" murine antibodies. This has been achieved by grafting murine Variable regions (VH and VL) which contain the Complementaritity Determining Regions (CDRs) responsible for antigen binding or the CDRs themselves onto the human antibody scaffolds. Alternatively, phage display has been employed for the screening of combinatorial libraries of human VH and VL genes, revolutionising antibody engineering (Watkins and Ouwehand, 2000). Two types of recombinant antibodies have been favoured, antigen binding fragments (Fab) or single-chain Variable region fragments (scFv) (Fig. 22). V gene repertoires from human B cells have given rise to libraries from immunised subjects and also naïve libraries of recombinant antibodies. Immune libraries are composed of antibody fragments which show a certain specificity to a given antigen. Screening this type of library selects high affinity binding antibodies. Naïve libraries are composed of antibodies of diverse specificity but lack somatic affinity maturation associated with the immune libraries. Screening of naïve libraries selects for novel specificities and the affinities of the isolated antibody fragments are in correlation with the complexity of the library (de Haard et al., 1999; Vaughan et al., 1996). The affinity of these antibodies is increased by molecular affinity maturation using techniques such as CDR mutagenesis (CDR walking mutagenesis), chain shuffling (combination of different VH and VL repertoires) or the substitution of the VH CDR3 by a synthetic sequence (Rader and Barbas, 1997; Watkins and Ouwehand, 2000). These techniques have generated antibodies with diverse (human) therapeutical applications such as tumour antigens (discussed later), viral antigens, for example, Human Immunodeficiency Virus (Kempf et al., 2001; Yang et al., 1995) and has aided the study of human autoimmune conditions such as anti-thyroglobulin antibodies in Hashimoto's thyroiditis (McIntosh et al., 1996).

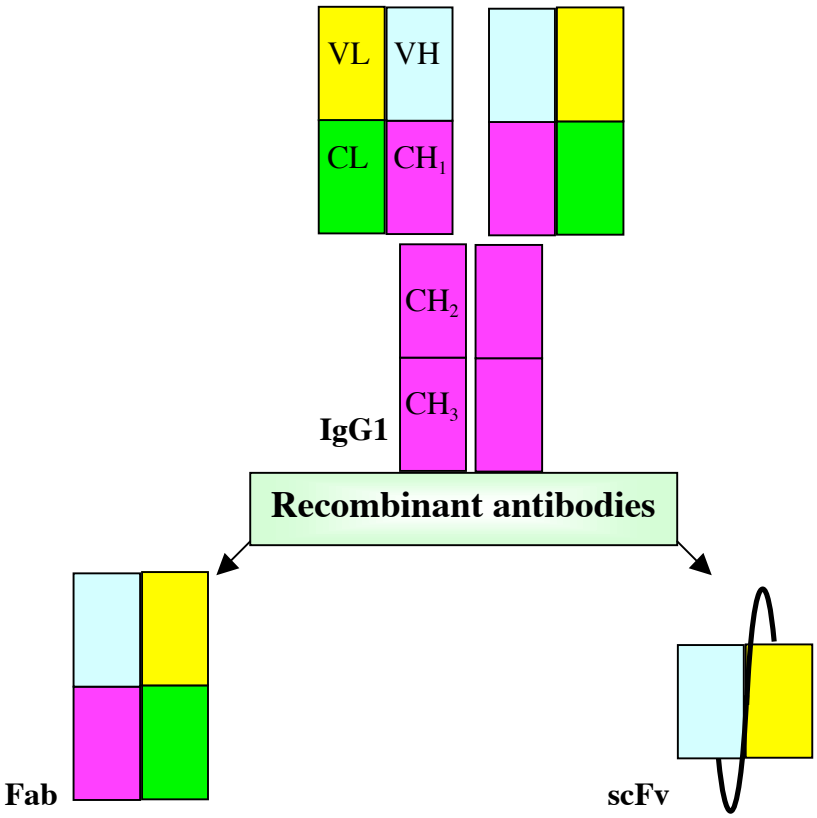


Figure 22.
Schematic representation of IgG1 and antibody fragments.
Antibody engineering allows the obtention of several antibody fragments. The antigen-binding fragment (Fab) and the single-chain variable fragment (scFv) represent the antibody fragments most frequently used in phage display libraries. scFvs are formed from the association of VH and VL in a continuous polypeptide by a flexible linker sequence composed of glycine and serine amino acids.

5.3.1.2 Enzymes

Directed evolution of enzymes is far more powerful than the rational design even in the light of exhaustive functional and structural data. Phage display has been applied for the evolution of both biophysical and functional properties of enzymes. One biophysical property which is of paramount importance in biotechnology is thermostability. Ruan and co-workers subjected the protease subtilisin prodomain, unfolded at room temperature, to directed evolution mediated by phage display (Ruan et al., 1998). After three rounds of biopanning, a mutant was obtained which was thermodynamically stable at room temperature. Another application is the modification of substrate specificity. For example, the specificity of the savinase (serine protease subtilisin 309) from the *Bacillus lentus* was modified by Legendre and co-workers using phage display (Legendre et al., 2000). Indeed, substrate preference of evolved enzymes was for charged residues at the P4 site whereas wild type enzymes show preference to hydrophobic residues at this site.

5.3.2.3 Peptide display

5.3.2.3.1 Antibody epitopes

The process of epitope mapping using phage display was essentially described by Scott and Smith in 1990 (Scott and Smith, 1990). Epitope mapping was one of the first descriptions of combinatorial peptide phage display libraries (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990). They created a library of linear peptides composed of 6 randomised amino acids which were displayed on the pIII of fd phage particles. For this they produced a degenerate codon sequence $(NNK)_6$ where N is the nucleotide G, A, T or C and K, is either G or T. These 32 nucleotide triplets reduce the redundancy of a 64 codon set while representing the codons of all 20 amino acids and the amber stop codon. For the 6 amino acids, this represents 32^6 or 10^9 individual sequences that were then cloned into the fUSE5 fd phage vector, electroporated into bacteria and expressed. The monoclonal antibodies used, A2 and M33, are specific to the peptide DFLEKI, an epitope of myohemerythrin protein. After three rounds of selection the peptides selected had the consensus **DFL**(**E** or A)WL. In addition to epitope definition, these results underlined the importance of the first three amino acids of the epitope for antibody binding which was noted to be in accordance with independent experimental data. This additional information obtained from phage-peptide display epitope mapping allows the delineation of antibody epitopes. Indeed, in our laboratory, we have used the complementary methods of overlapping peptides, alanine scanning, X-scan and phage-peptide display for the determination of the physico-chemical properties of the amino acids at each position in the linear epitopes of two monoclonal antibodies (Choulier et al., 2002). One advantage of the phage display for this application is the rapidity and low cost with which the results are generated when compared with the synthesis of numerous peptides and their investigation using immunoassay techniques. We used two phage-peptide libraries displaying random linear peptides of 7 or 12 amino acids in length fused to the pIII of phage M13 and performed the biopanning on antibody immobilised on protein A or G-sepharose beads. The consensus motif generated by the phage-peptide display experiments on the mAb 6F4 in (Choulier et al., 2002) allows description of physico-chemical characteristics of the amino acids within the epitope. For example, it shows the importance of aromatic residues at position 9, the critical Q residue at position 10 and the unimportance of E at position 14 in binding. However, discrepancies between the systems were found, one explanation is the fundamental difference between the peptide-display which selects for best binders and the other non-combinatorial approaches used.

Although both the above examples study linear epitopes, discontinuous antibody epitopes have also been defined. For example, Birkenmeier and co-workers (Birkenmeier et al., 1997) describe the non-continuos epitope of the antibody raised against α2-macroglobulin using a linear hexamer peptide phage display library. However, some authors suggest that the use of constrained peptides may be important for the definition of discontinuous or structural epitopes. Constraint is obtained by a disulphide bond between flanking cysteine residues, forcing the peptide into a loop configuration. This may force configurations where, for example, otherwise buried hydrophobic residues are exposed (Ladner, 1995). Furthermore, the use of constrained peptides for this application is possibly advantageous as structure could be a prerequisite for high-affinity binding (Sidhu et al., 2003). Indeed, Vanhoorelbeke and co-workers used two phage libraries displaying linear pentadecamer and cyclic hexamer peptides for to determine the discontinuous epitope of an antibody raised against the human von Wilebrand factor (Vanhoorelbeke et al., 2003). They obtained homologous peptides from both libraries, and, importantly, selected cysteine-constrained peptides from within the linear library. Furthermore, Hoess and co-workers used phage-peptide display on a monoclonal antibody directed against PAI-1, a protease inhibitor (Hoess et al., 1994). They constructed both a linear and a constrained library of hexapeptides presented on the pIII of M13 phage and used a mix of these two libraries for biopanning experiments. The sequences isolated where all from the constrained library and gave rise to the consensus sequence CXRLXRSC which matches the amino acids 115 to 119 of the PAI-1 whereas un-constrained phage-peptides bound 50-fold less well.

5.3.2.3.2 Enzyme ligands

Enzymes have evolved to bind small molecular entities and it is not surprising that peptide display has allowed the selection of peptide inhibitors or substrates (Szardenings, 2003). For example, the screening of peptide libraries on alcohol dehydrogenase of *Saccharomyces cerevisiae* generated peptide inhibitors with μM affinity (Hyde-DeRuyscher et al., 2000). Selection of enzyme substrates of, for example proteases, requires an original approach. Indeed, methodology has been devised where a module that allows affinity retention is displayed on phage-particles via a random peptide. After incubation with the target enzyme, the library is passed on the appropriate affinity matrix corresponding to the tag. When the enzyme has cleaved the peptide linker, the corresponding phage are not retained on the affinity matrix allowing the selection of particles displaying the desired peptide substrates (Nilsson et al., 2000). This has been applied to Subtilisin BPN and Factor Xa using human Growth Hormone (hGH) as the tag and anti-hGH antibodies in the affinity matrix (Matthews and Wells, 1993)

5.3.2.3.3 Intracellular protein interactions

Intracellular protein functions are often brought about by domains that interact with continuous linear stretches of amino acids of their protein partners. Epitope mapping using combinatorial peptide libraries as described for antibodies has been applied to these proteins (Pelletier and Sidhu, 2001). As will be discussed, protein domains which have evolved to recognise linear stretches of amino acids, and may form a structurally homologous family (for instance SH2 or 3 and PDZ domains) or be unique to the protein (for instance the p53-binding MDM2 domain) (Kay et al., 2000).

The PDZ domains are composed of up to 100 amino acids that recognise and bind the C-termini of a multitude of proteins (possibly as many as 260) assembling multi-protein complexes at subcellular sites (Sidhu 2003). Type I PDZ domains recognise free carboxy terminal core motif X S/T X V/I/L and type II, XΦXΦX, additional specificity is obtained from up to 6 C-terminal amino acids. As discussed previously, the technical hurdle of C-terminal display using phage vectors restrained the use of phage display in exploring these domains as these require free C-termini to bind. However, M13 C-terminal display has been recently used to investigate the PDZ domain of ErbB2-interacting protein, Erbin and allowed the definition of novel protein partners. Indeed, phage-peptide display provided a consensus sequence D/E T/S W V that found to be distinctly different to the DVPC C-terminal sequence of ErbB2. Searching genomic databases found that this sequence was similar to the DSWV C-terminal sequence of three homologous catenin proteins. Ensueing *in vivo* and *in vitro* studies confirmed that Erbin bound these catenins through their C-terminus (Laura et al., 2002).

An example of a unique linear interacting domain mapped by phage display is p53-binding region of the MDM2. Böttger and co-workers used phage-peptide display to isolate peptides that bound to hdm2 protein and found the consensus motif XFXDXWXXLX which coincided with the N-terminal p53 sequence ^{18}T**F**S**D**L**W**K**LL**P^{27} (bold letters indicating identical residues) (Bottger et al., 1997; Bottger et al., 1996). It was already known that this region was important for the binding of MDM2 to p53 (Picksley et al., 1994) and that L22 and W23 were critical for this interaction (Lin et al., 1994). However, further structural studies of MDM2 bound to a 15-mer wild-type p53-derived peptide revealed a hydrophobic cleft to which the peptide binds as an α-helix and it was noted that F19, W23 and L26 insert deep into this groove (Kussie et al., 1996), the same amino acids highlighted by the consensus sequence derived from the phage-peptide display experiments. Using these data, Garcia-Echeverria and co-workers rationally designed antagonists of the MDM2-p53 interaction (Garcia-Echeverria et al., 2000).

5.3.2.3.4 Tumour targeting

Modern therapeutical strategies aim to incorporate a greater level of specificity either by targeting tumour-specific intracellular components or the tumour cells themselves. Strategies targeting tumours take advantage of tumour-specific modifications in the cellular environment (extracellular matrix, vasculature) or the molecular landscape of the tumour-cell surface (Kim and Toge, 2004; Nilsson et al., 2000). Specific cellular markers can be used to distinguish between cell type in an organ-and/or tumour-dependant manner. Indeed, tumourigenesis induces modification of expressed cell-surface marker and, expression of cell-surface markers distinguishes the angiogenic endothelial cells within tumours from established vessels and even normal angiogenic endothelial cells. Therefore, type-specific cell-surface markers may be used for tumour-targeting.

Several antibodies targeting cellular receptors for the treatment of tumours have been approved for clinical use with different levels of functional activity. For example, antibodies inhibiting the function of a receptor (IMC-C225 cetuximab, an anti-epidermal growth factor receptor mAb) or deliver a toxic payload (Mylotarg, an anti-CD33 mAb is conjugated to the calicheamicin toxin and Zevalin, an anti-CD20 mAb, is conjugated to a radio nucleotide) (Aina et al., 2002). A bobesin/gastrin releasing peptide was used for the treatment of prostate, breast and pancreatic cancer and Octreotide, a somatostatin analogue was used for imaging of somatostatin receptor positive tumours (Landon and Deutscher, 2003). These peptides are ligands of known over-expressed molecules at the surface of tumour cells and have been identified by rational design. This approach is not only restricted to known tumour antigens but is time-consuming. Combinatorial libraries of peptides, antibody and antibody fragments have been screened for tumour targeting and have given rise to tumour ligands of known proteins (purified and ectopically expressed in cultured cells). Alternatively, biopanning has been successfully performed on whole cells, organs and, under certain conditions, *in vivo* without prior knowledge of the surface landscape (Aina et al., 2002; Landon and Deutscher, 2003; Nilsson et al., 2000).

The first recombinant antibody derived from a phage display repertoire for tumour-targeting was the scFv MFE-23 raised against carcinoembryonic antigen. This antibody fragment has been demonstrated to bind more efficiently than hybridoma-derived whole antibody raised against the same antigen. The clinical use of this fragment (Fab, scFv) has been in the specific detection of liver metastases in patients with colorectal cancer (Nilsson et al., 2000). The therapeutical use of antibodies is however limited by their size,

immunogenicity and bioavailability (for systemic applications) (Aina et al., 2002). Peptides, however, are less prone to such problems and are used in tumour targeting and methods have been developed for the selection within phage-peptide libraries which target either *in vitro* cultured tumour-derived cells or tumour vasculature *in vivo* (Aina et al., 2002; Brown, 2000; Nilsson et al., 2000).

In vivo screening of vasculature using phage display libraries requires systemic injection of the library and recovery of phage from tissue sections or organs. This approach has revealed the molecular heterogeneity of vascular endothelium (Rafii et al., 2003), and has led to the isolation of peptides demonstrating binding specificity to organs and tumours in mice and, more recently, in a human patient (Arap et al., 2002). The selected peptides target integrins, matrix metalloproteases and vascular growth factor receptors (reviewed by (Rafii et al., 2003)). The methodologies applied in *in vivo* vasculature screening favour simple binding of phage (Michon et al., 2002; Molenaar et al., 2002). However, integrin-binding peptides are known to induce cellular internalisation of viral vectors and filamentous phage (Ivanenkov et al., 1999a) and have been used by both Arap *et al*. and Ellerby *et al*. to address pro-apoptotic peptides to angiogenic tumour endothelial cells (Arap et al., 2002; Ellerby et al., 1999). Furthermore, tumour-specific integrin-targeting peptides have also been used to direct the antiangiogenic cytokine interleukin 12 and gene therapy vectors (for example, adenovirus and adeno-associated virus) (Trepel et al., 2002). Vasculature targeting does not address the tumour cells directly but by causing vascular destruction, prohibiting neovascularisation by creating a hostile environment for the tumour which results in necrosis of the tumour cells (Arap et al., 2002).

Direct targeting of tumour cells using phage display was, until recently, limited to *in vitro* methods (Aina 2002). However, Landon and Deutscher have recently described the isolation of tumour cell targeting peptides from an *in vivo* systemically circulating phage library cleared of endothelial binding clones (Landon and Deutscher, 2003). Interestingly, when the cells used to generate the mouse xenograft model where cultured *in vitro* the phage-peptides were found to bind and internalise these. Transcytosis of phage-peptides has been demonstrated (Ivanenkov and Menon, 2000; Samoylova and Smith, 1999) but this is the first description of a systemically circulating tumour cell-targeting phage-peptide.

6. Other genotype-phenotype methods used for screening combinatorial libraries

6.1 Two-hybrid systems

In two-hybrid systems, screening is performed *in vivo* and allows protein-protein interactions to be investigated. The principle, first described by Fields and Song (Fields and Song, 1989), resides in the separation of the regulation and DNA-binding domains of a transcription factor, rendering it inactive. Genetically, the protein of interest is fused to one of these moieties and the combinatorial library to the other. Interaction between a member of the library and the protein of interest brings about the formation of a complex that activates the transcription of a reporter gene. Initially described in yeast (*Saccharomyces cerevisiae*), two-hybrid systems and those derived from the same principle, have been adapted to be used in bacteria (Hu et al., 2000) and mammalian cells (Fearon et al., 1992). These systems have been applied to increasingly complex strategies to analyse interactions between proteins and molecules of biological interest including other proteins, RNA, pharmacological molecules and peptides (Serebriiskii et al., 2001). Depending on the application, both the advantage and, paradoxically, disadvantage of these systems is that selection occurs *in vivo*. Indeed, for example, intracellular expression of secreted proteins such as antibodies in eukaryotic cells fails to produce functional, soluble and monodisperse molecules (Sibler et al., 2003) whereas for intracellular proteins which have been found to be difficult to express and purify from bacteria such as the E6 oncoprotein of Human Papilloma Virus 16 (Nomine et al., 2001a; Nomine et al., 2001b) have been used to screen peptide libraries (Butz et al., 2000; Elston et al., 1998). Other limiting factors associated with the use of transcription factor domains, for example when using proteins with DNA-binding domains, may be treated by the use of the reconstitution of enzyme domains (Pelletier and Sidhu, 2001). Additionally, construction of two-hybrid combinatorial libraries suffers from the limiting transformation step which restrains diversity to approximately 10^9 individuals.

6.2 Bacteria display

Bacteria display is a surface display system homologous to the phage display system. In these systems, peptides and proteins have been genetically fused to bacterial proteins and

displayed at the surface of bacteria (gram negative and positive). Although display of functionally defined molecules has been favoured over combinatorial libraries (Benhar, 2001), enzyme (Olsen et al., 2000) and peptide (Lu et al., 1995) combinatorial libraries have been screened using this technology. The diversity of these libraries is again restrained by the required transformation of the bacterial cells. However, one interesting advantage of bacterial display is that the displaying bacteria library may be screened using Fluorescence-Activated Cell Sorting (FACS) (Olsen et al., 2000) which is not possible for phage display systems as the displaying particles are too small.

6.3 Ribosome and mRNA-protein display

Reviewed by Jermutus 1998 and first described by Mattheakis *et al.*, Nemoto *et al.* and Roberts and Szostak, ribosome display and mRNA-protein display are two homologous techniques for *in vitro* selection of combinatorial libraries. These are homologous techniques as they are constructed by physically linking the mRNA to the polypeptide it encodes, they differ in their construction. However, these techniques present the advantage of not relying on the limiting transformation step for the creation of libraries and can compose up to 10^{13} individuals (Mattheakis et al., 1994; Nemoto et al., 1997; Roberts and Szostak, 1997).

Ribosome display takes advantage of persistent ribosome association with the mRNA and the nascent polypeptide in the absence of a stop codon. Alternatively, mRNA-protein display uses DNA/puromycin-modified mRNA for translation that results in the association of the nascent polypeptide with the puromycin and the encoding mRNA. Following selection, mRNA is amplified by PCR. These techniques have been successfully applied to the epitope mapping of antibodies (Jermutus et al., 1998), the evolution of biophysical properties of proteins such as antibodies (Jermutus et al., 2001), and the screening of synthetic antibody library (Hanes et al., 2000). However, they suffer from a major disadvantage as these technologies use “naked” RNA that requires an RNase-free environment. This fragility may be of great hindrance for certain applications for which the more robust phage-display techniques is better suited.

RESEARCH PROJECTS

7. Article A

7.1 Scientific Context

Treatment of cervical cancer depends on the stage of tumourigenesis. For example, cervical intraepithelial carcinoma (stage 0) requires only local surgical procedures, whereas invasive carcinoma (stages I to IVb) requires radiotherapy, chemotherapy and radical surgical procedures (NCI, 2003b). These methods suffer from a lack of specificity for the tumour cells. Chemotherapy, for example, relies on the preferential accumulation of toxic molecules in proliferating tumour cells and the high doses required for remission results in toxicity to normal cells. To address this, two types of vaccines have been developed for the targeted treatment of cervical cancer. Therapeutic vaccines which induce targeted cellular immunity towards HPV-infected epithelial cells and prophylactic vaccines which induce virus targeting antibodies which neutralise new but not established infection are currently undergoing clinical trial. Therapeutic vaccines target HPV E6 and E7 oncoproteins continuously expressed in established lesions whereas prophylactic vaccines utilise virus like particles spontaneously generated from the L1 capsid proteins and in some cases the L2 capsid protein as well (Tjalma et al., 2004). Tumour-targeting ligands have yet to be characterised for cervical cancer and could be used to develop targeted therapeutical strategies in complement to therapeutical vaccines. Our objective in Article A was therefore to isolate tumour cell-targeting peptides for the development of therapeutical strategies for cervical cancer.

7.2 Introduction and rational of experimental design

Cellular internalisation is a prerequisite of many modern therapeutical strategies as these are based on the intracellular delivery of peptides, polypeptides or genetic material by viruses, liposomes, proteins or polymers. Therefore, it is favourable that tropism modification of these vehicles is obtained by ligands that are internalised most efficiently. In biopanning, where phage binding is the selection criterion, resulting ligands target the most abundantly expressed cellular receptors but not specifically those that undergo internalisation.

Although peptides selected for binding cellular receptors have been used to internalise cells, for example integrin-binding peptides defined by Arap *et al.* and Ellerby *et al.*, Ivanenkov *et al.* describe methods for the isolation of phage-peptides specifically for cellular internalisation (Arap et al., 2002; Ellerby et al., 1999; Ivanenkov et al., 1999a; Ivanenkov et al., 1999b). The authors demonstrate protocols which effectively only allow enrichment of intracellular phage by protease inactivation of extracellular particles. Furthermore, the use of a phagemid-based library and extensive multivalent presentation of peptides on the pVIII of M13 potentiates the selection of intracellular phage. The phagemid particles produced are only 500 nm in length (compared to 900 nm wild-type M13 phage) and despite demonstration of successful internalisation of phage particles (Arap et al., 2002; Larocca et al., 1999), this may indeed facilitate internalisation. Phagemid-based particles are smaller in size than their phage counterparts as the size of the phage particle and thus the number of pVIII present in the coat, is directly dependent on the of the encapsidated genetic marterial. Indeed, for every nucleotide of the genetic material, 0.42 copies of the pVIII are required and therefore, the display valency and size of the displaying phage particle is defined by the system used to produce them. It has also been noted that multivalent presentation of ligands (peptides or antibodies) increases internalisation capability of phage and phagemid particles (Ivanenkov et al., 1999b; Legendre and Fastrez, 2002; Poul et al., 2000) as, independently of receptor oligomerisation, multivalent presentation may allow the recruitment of several receptors, facilitating internalisation (Ivanenkov et al., 1999b). Although the precise nature of phage particle endocytosis has yet to be elucidated, Ivanenkov and co-workers demonstrated that phage-peptides selected specifically for internalisation penetrate cells 100 times more rapidly than integrin-binding phage-peptides (Ivanenkov et al., 1999a; Ivanenkov et al., 1999b).

To isolate peptides for cervical cancer targeting we performed biopanning using a similar method to that described by Ivanenkov *et al*. on the established cell line SiHa derived from a cervical carcinoma transformed by HPV16. These cells were favoured as HPV16 is the causative agent in the majority of cervical cancer (Jordan and Monaghan, 2004). Additionally, we used the same phagemid library as Ivanenkov, the pC89 CX9C. As described by Felici *et al*., the 9 random amino acid library is fused to the pVIII of M13 (Felici et al., 1991). The peptides are presented constrained into a loop by a disulphide bond formed between flanking cystein residues. The library was constructed using 26 completely randomised nucleotides and at least 25% of the 9.4 x 10^7 transformants gave clones producing recombinant protein without any in-frame non-sense codons (Ivanenkov et al., 1999b). Thus, the theoretical efficient diversity of the library is 2.35 x 10^7 individual sequences It was furthermore ascertained that on each phagemid particle, between 30 and 300 copies of the pVIII fusion was present (Felici et al., 1991).

7.3 Article A

Identification using phage display of peptides promoting targeting and internalisation into HPV-transformed cell lines

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7.4 Discussion

7.4.1 Specificity

Specificity of the selected phage is, within the limits of the cells tested in article A, restricted to HPV-transformed cells. This demonstrates the efficiency of the biopanning, as, prior to selection, subtraction of the phage library on non-HPV-transformed cells was not performed. Indeed, it has been documented that phage selected in such a non-biased manner without subtraction show 10-100 fold-increased specificity for targeted cells (Brown, 2000). From experimental evidence, the selected peptides may target more than one receptor. Indeed, it is demonstrated that γ6 phage-peptide is specific of only SiHa cells (Article A, Fig.1), whereas the α1 and β5 phage-peptides show specificity for both CaSki and HeLa cells. Therefore, γ6 peptide may target a receptor that is much less expressed on SiHa cells and not at all on the other cell types. The observed specificities indicate that the targeted receptors are greatly expressed on HPV-transformed cells and although as yet unidentified, these must play a biological function. Although evidently rare, these may be expressed on other cells but to a much lower extent.

7.4.2 Constraint

An interesting point to underline here before treating questions regarding affinity is the relative frequency of the selected clones that are present in the final phage population after three rounds of selection (Article A, Table 1). Cellular internalisation preceded by binding is the selection criterion of this biopanning, therefore, it is possible to envisage that selection favoured poor affinity for a receptor with a high rate of internalisation. The resulting frequencies give a poor indication of binding affinity and may give an indication of internalisation capability. The relatively high representation of the α sequences may, for example, be due to a more easily expressed DNA sequence in bacterial cells.

It has been observed that constraint by a disulphide bond between flanking cystein residues increases affinity of a peptide ligand for its target (Ladner, 1995). It is argued that constraining a peptide may force exposition of side chains that may be buried in a linear peptide. However, Mazzucchelli and co-workers isolated homologous peptides from

constrained and non-constrained peptide libraries by performing biopanning for human neutrophil targeting (Mazzucchelli et al., 1999). It is interesting therefore that, when the flanking cysteine residues of the selected peptides were replaced by serines producing linear peptides, only β5 phage-peptide remained functional (Article A, Fig. 2). An explanation of this may be that β5 peptide may adopt, both in the absence and presence of flanking cysteine residues, the same conformation presenting only a minority of the amino acids for contact with the target, the others forming a substructural scaffold for their display. The affinity of the selected peptides for their respective receptors is an interesting field of investigation and will be greatly aided by the definition of the targeted receptors. However, with respect to their conformational requirements, article A defines the functional limitations for the selected peptides that will be of importance in their application to tumour targeting.

7.4.3 Valency and presentation

7.4.3.1 Multivalent phagemid particle presentation

As discussed in the Introduction of Article A (section 7.2), multivalent presentation of displayed ligands potentiates phage and phagemid-derived particle internalisation by either the virtue of receptor dimerisation or their extensive recruitment. Furthermore, internalisation may be aided by the use of phagemid vectors. Indeed, this notion may be supported by previous work performed in our laboratory by Dr Georges Orfanoudakis using the Ph.D. 12 library (New England Biolabs). This is a phage library where 12 random and non-constrained amino acids are displayed as fusions to all 5 pIII proteins of M13. In similar experiments to that detailed in Article A and after three rounds of biopanning, an enrichment of only 10 fold was obtained. Enrichment is a direct result of cellular internalisation, the selection criterion. When compared to the 10^5-fold enrichment obtained in article A, this demonstrates a failure of the biopanning process.

7.4.3.2 Monovalent GFP presentation

For their use in tumour homing strategies it was important to determine whether the targeting function of the peptides isolated in article A was maintained outside the phage environment and for this we chose the C-terminus of the reporter protein EGFP which would allow us to follow peptide-mediated binding and internalisation. All three peptides induced internalisation of the EGFP into SiHa cells (Article A, Fig. 4), demonstrating the rigour of their targeting capability. Firstly, monovalent presentation can therefore induce internalisation of small macromolecules, this suggests that the multivalent presentation apparently required for rapid internalisation may be due to the relatively large size of the phage and phagemid particle which requires recruitment of several receptors to possibly create a large coated pit. Secondly, the selected peptides retain their targeting capacity when presented as C-terminal fusions. This capacity was probably aided by the conformational constraint imposed by the flanking cysteine residues (Ladner, 1995).

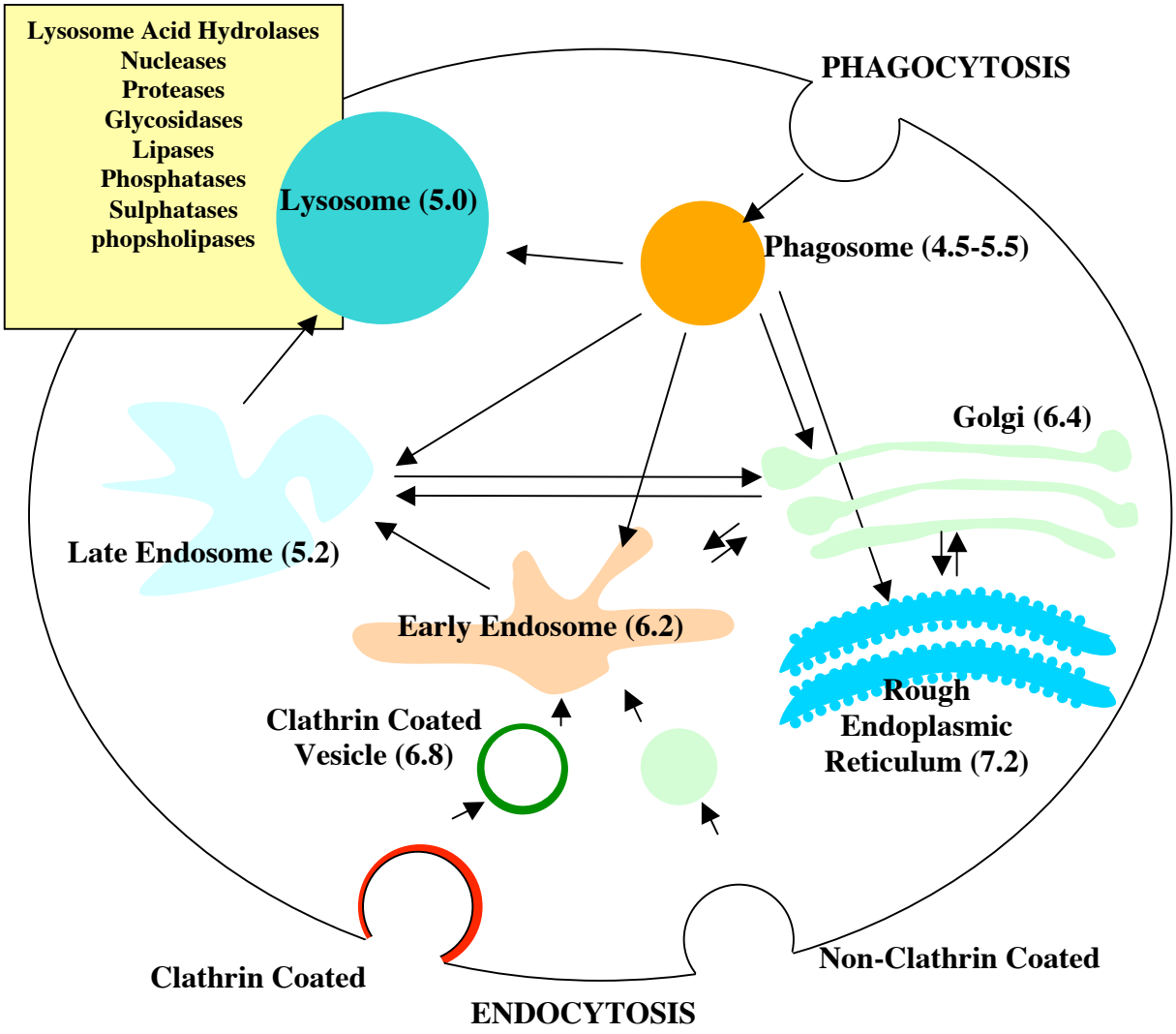

Figure 23.
The pathways followed by internalised material.
Both endocytotic and phagocytotic pathways can communicate along the same intracellular pathways. Degradation takes place in the lysosome which contains an extensive range of acidic hydrolases. The pH of individual compartments is indicated between brackets.
Adapted from Faundez and Hartzell 2004, Griffiths 2004, Maxfield and McGraw 2004 and Gruenberg and Stenmark 2004.

7.5 Perspectives: Peptide-mediated delivery of therapeutic agents

Extracellular material is endocytosed into cells via several pathways, including phagocytosis, coated pits (clathrin and caveolae), non-coated pits and macropinocytosis. Size as opposed to receptor determines the pathway followed by different cargos. Indeed, Reiman *et al*. have used latex beads of various sizes to demonstrate that clathrin-mediated endocytosis has a 200 nm size limit and that the caveolae-mediated pathway is favoured for beads of up to 500 nm (Rejman et al., 2004). Whereas beads of 1 μm are readily internalised into specialised cells such as macrophages by phagocytosis, these can be internalised by all cells (Griffiths, 2004). However, once inside the cells, internalised cargo may communicate along the extensive endocytotic vesicular system by vesicle fusion or budding, (Fig. 23) (Griffiths, 2004; Maxfield and McGraw, 2004; Nabi and Le, 2003). As discussed previously, both M13 phage particles which are approximately 900 nm in length and phagemid derived particles used in Article A which are approximately 500 nm in length have been found to be internalised into cells and Ivanenkov *et al*. proposed that multivalent presentation and a smaller size may facilitate internalisation (Ivanenkov et al., 1999a). These particles may indeed be sufficiently small to utilise the caveolae-dependant pathway, alternatively multivalent presentation may facilitate phagocytosis.

As can be seen in Fig. 4 of Article A, EGFP-peptide fusion proteins are endocytosed and partially co-localise with transferrin. The mode of entry of transferrin has been extensively studied. It binds its cellular receptor that is internalised through clathrin-coated pits, the iron it carries is released in the early endosome and the apo-transferrin is recycled back to the cell surface and transferrin is a marker of early, sorting and recycling endosomes (Maxfield and McGraw, 2004). However, internalised EGFP-peptide is sorted into the late endosome/lysosome pathway as chloroquine, a lysosomotropic agent, was required to protect internalised EGFP from degradation. Indeed, the lysosome is the principle site of intracellular digestion. In the sorting endosome, material destined for degradation is sorted into endosomal carrier vesicles (or multi vesicular bodies / endosomes) that move material to the late endosome which inturn fuses with the lysosome (Gruenberg and Stenmark, 2004). The lysosome contains an extensive collection of hydrolases with broad specificity (Fig. 23). There are, however, peptides which target receptors that are sorted into different pathways and have been used for the delivery of proteins to the cytosol of cells for example the HIV-Tat peptide. This peptide along with, for example, Antennapedia homeodomain and Herpes Simplex Virus type 1 VP22 protein was long thought to act as a Protein Transducting Domain (PTD). Indeed, it was heralded that these were revolutionary protein domains that could mediate protein transduction across cellular membranes without using receptors and thus bypassing endocytosis (Gariepy and Kawamura, 2001). However, in the case of HIV-Tat at least, it is assumed now that it binds to cellular receptors (VEGFR-2 or Flk-1/KDR) and immunofluorescence results supporting the PTD hypothesis have been considered to be artefacts of the method used for the fixing of cells (Olsnes et al., 2003). In conclusion, any material delivered to cells using the peptides isolated in Article A will ultimately enter the lysosome for degradation. The intracellular therapeutical strategies developed using these peptides should provide an endosomal escape method.

7.5.1 Bacterial toxins, drugs and pro-apoptotic peptides

7.5.1.1 Bacterial toxins and drugs

Targeting peptides may be used directly associated to the therapeutical molecules. For example the drug doxorubicin has been coupled to a transferrin receptor-targeting peptide (Schatzlein et al., 2001), proapoptotic peptides have been fused to vascular targeting peptides (Arap et al., 2002) and several bacterial toxins have been fused to the neuropeptide Substance P (Lappi and Wiley, 2000). The use of diphtheria toxin conjugates has been the most recent subject studied in our laboratory and is extensively discussed in the section 8.

7.5.1.2 Pro-apoptotic peptides

These peptides are antibiotic peptides that do not affect eukaryotic cells, in the concentrations used. Unstructured in an aqueous environment, a sub-type of antibiotic peptides form disruptive α-helices in amphipathic media including cell membranes (Javadpour et al., 1996). The principle characteristic of such peptides is that they are linear and amphipathic having hydrophobic and cationic residues down each side of an α-helix. These peptides target the anionic phospholipid head groups. Thus, they preferentially disrupt the negatively charged cytoplasmic membranes of bacteria and mitochondria which have high transmembrane potential whereas they do not disrupt eukaryotic membranes, composed of zwitterionic phospholipids and which have a low transmembrane potential (Ellerby et al., 1999). When in the cytosol of cells, these peptides disrupt the mithochondrial transmembrane potential and induce the release of cytochrome C inducing the caspase cascade and apoptosis. The peptide of sequence $(KLAKLAK)_2$, constructed by Javadpour and co-workers through *de novo* design (Javadpour et al., 1996), has been successfully used to specifically induce apoptosis in endothelial cells when coupled to an integrin-targeting peptide both in culture (Ellerby et al., 1999) and *in vivo* (Arap et al., 2002). In our laboratory, the D-amino acid peptide $(KLAKLAK)_2$ were coupled to L-amino acid α1, β5 and γ6 constrained peptides. These chimeras are currently undergoing investigation.

7.5.2 Targeting vehicles (non-viral and viral vectors)

Tumour targeting peptides have been associated with a variety of delivery vehicles that can deliver therapeutical molecules such as drugs or genetic material. Examples include liposomes, synthetic polymers, proteins and viruses.

7.5.2.1 Liposomes

Liposomes have gained clinical approval and are considered a mainstream option for the delivery of anti-cancer drugs and genetic material such as expression vectors or anti-sense RNA (Brignole et al., 2003). Liposomes are phospholipid bilayer vesicles that can form around therapeutic drugs for their cellular delivery and have been used as cancer therapeutics. Using "stealth liposomes" which are coated in a hydrophilic carbohydrate of polymer that increases the circulating half-life, tumour targeting is passive, relying on the increased permeability of tumour vasculature for accumulation in a manner analogous to chemotherapy. More recently, targeting moieties such as antibodies, antibody fragments and peptides have been used to increase their targeting potential (Sapra and Allen, 2003).

7.5.2.2 Synthetic polymers and proteins

A widely used polymer for cellular transfection is polyethylenimine (PEI). This condenses the DNA to be transfected into aggregates that are internalised. In the endosome, the PEI acts as a proton sponge which results in rupture of the endocytotic vesicle (Ferrari et al., 1999). PEI has extensive tropism but can be modified by targeting peptides such as integrin-binding RGD peptides (Erbacher et al., 1999a; Erbacher et al., 1999b) DNA-binding domains of proteins have been used for transduction and have also been modified by targeting ligands. Such strategies generally incorporate an endosomal escape mechanism, for example proteins of viral origin or inactivated virions and bacterial toxins (Uherek and Wels, 2000).

7.5.2.3 Viruses

Gene therapy is the cellular delivery method of a gene often encoding a therapeutic protein. Viral vectors have been favoured due to their relatively high gene transfer capacity that is directly related to the level of transfection. The viruses employed for gene therapy include adeno and adeno-associated virus, vaccinia virus and human simplex virus. These viruses have extensive natural tropism and therefore have been the subject of targeting modifications, termed transductional re-targeting (Wu et al., 2002). In our laboratory, François Deryckère in collaboration with Dan Von Seggern and Glen Nemerow of the Scripps Research Insitute, have successfully retargeted adenovirus 5 using the α1, β5 and γ6 peptides. This was achieved by concomitant modification of the fibre knob, the globular protein of the viral capsid and which is responsible for tropism through binding to its principle cellular receptor, Coxsackievirus-Adenovirus Receptor (CAR). CAR-binding was ablated by two point mutations in the AB loop and the peptides, α1, β5 or γ6, were introduced into the HI loop. These modified virions demonstrate preferential tropism for HPV-transformed cultured cells. It is envisaged that re-targeted adenovirus could be used for the delivery of therapeutic genes and for tumour-dependent oncolysis.

An interesting development in gene therapy vectors is the use of targeted phage particles. Pioneered by David Larocca, this has been a field of growing interest as phage have little or no tropism for human cells, easily allow the presentation of targeting ligands and can be produced at high titres in bacteria. However, they have poor transduction (gene delivery) efficiency. One factor may be that they lack the capacity for post-internalisation escape from the endosomal system and nuclear localisation of genetic material (Larocca et al., 2002). Such mechanisms are inherent in viruses that are employed for gene therapy and the future development of phage-mediated transduction relies on progress in this area.

7.6 Perspectives: Defining the cellular receptor(s) targeted

The peptides isolated in Article A are ligands of undetermined cellular receptors. Bibliographic research has provided little insight into possible candidates (Fujimoto et al., 2004) although Schulz and co-workers demonstrate that a sub-group of cervical and endometrial cancer cells express somatostatin receptors (Schulz et al., 2003). However, somatostatin is a constrained cyclic peptide of sequence, AGCKNFFWKTFTSC that bares little resemblance to the isolated peptides. Therefore, the isolation and characterisation of the receptors targeted by these peptides will require the production of a cDNA expression library of SiHa cells and to transfect this into a cell line not targeted by the phage-peptides. It is envisaged that fluorescently labelled phagemid particles or free peptides will be used for clonal selection will allow identification of the targeted receptors.

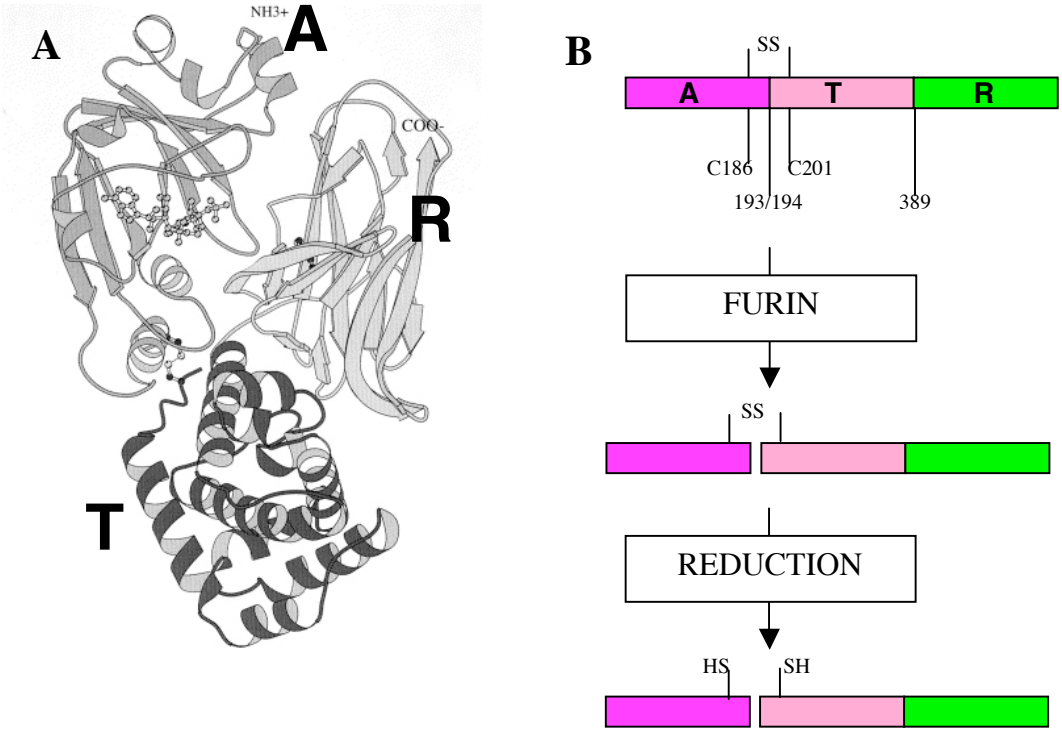

Figure 24.
Representations of the structure of the diphtheria toxin.
(A) The 3D representation of DT structure reveals the three discrete folding domains, the catalytic A domain (also named Catalytic, C-domain), the Translocation, T-domain and the Receptor-binding, R-domain. (B) Holotoxin is synthesised as a single polypeptide, which is nicked by the protease furin which specifically cleaves the peptide bond between amino acids at positions 193 and 194. Holotoxin is maintained by a disulphide bond formed between the cysteine residues at positions 186 and 201. Reduction of this disulphide bond in nicked protein results in separation of the two polypeptides.
Adapted from Colier 2001.

8. Diphtheria toxin

8.1 Introduction

As is described in Article A, the selected peptides drive internalisation of EGFP which then follows the endosomal pathway which results in degradation in the lysosome. This represents the major limiting-step for the application of the peptides to the delivery of therapeutic molecules to targeted cells is the cellular endosome-lysosome system. Cytosolic escape from this system has been addressed by naturally occurring pathogenic entities such as viruses and bacteria. Indeed, a number of bacterial toxins have been studied for their transducing capacity as these toxins are endocytosed via cellular receptors and transduce their toxic moiety into the cytosol of cells, effectively escaping the endosomal system. For example, the cholera and Shiga toxins are translocated into the cytosol by endoplasmic reticulum-associated degradation following retrograde transport through the Golgi apparatus, a method appropriated from miss-folded proteins. However, a more direct route is taken by toxins which contain specialised translocation domains, the most studied of these is the Diphtheria toxin (Olsnes et al., 2003; Schiavo and van der Goot, 2001).

Part of the AB bacterial toxin family which is composed of an enzymatic A component translocated into the cytosol by the B component across the endosomal membrane (Menetrey et al., 2005), the diphtheria toxin (DT), produced by *Corynebacterium diphtheriae,* is the most studied of the A1B1rt type (which also includes: exotoxin A and botulinum neurotoxin). The A1B1rt toxins have a B component which consists of two functionally and structurally independent domains: receptor binding (R) and translocation (T) (Menetrey et al., 2005) (Fig. 24). This configuration has allowed the globular receptor-binding domain (R) of DT to be replaced with a host of targeting molecules such as antibodies, interleukins and peptides for tumour targeting (Frankel et al., 2002).

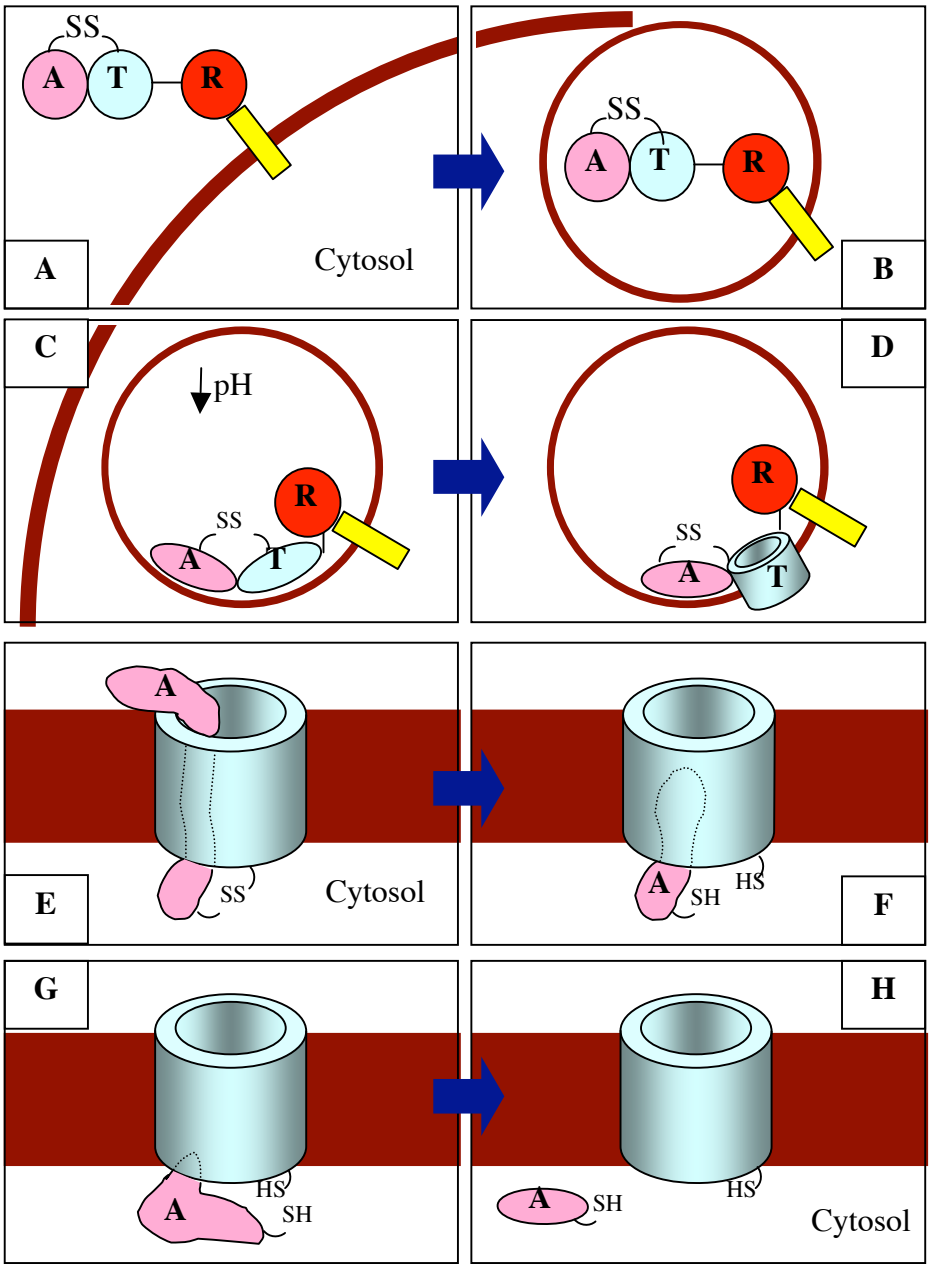

Figure 25.
Internalisation of diphtheria toxin and translocation of A-domain.
Schematic illustration of the chaperone model for translocation. (A) R-domain binds to the cellular receptor (B) inducing endocytosis. (C) Acidification of the endosomal environment (D) causes conformational change in the AT-domain. (E) The T-domain creates a "pore" in the endosomal membrane and the A-domain adopts a molten globule conformation that is translocated through the T-domain "pore". (F) The disulphide bond linking the A and T domains is reduced in the cytosol (G and H) allowing complete translocation of the A domain into the cytosol.
Adapted from Hammond et al. 2002.

The receptor binding domain of the naturally occurring DT binds the EGF-like domain of the heparin-binding epidermal-growth-factor-like growth factor precursor (HBEGF precursor) which itself associates with CD9 and heparin sulphate proteoglycan at the plasma membrane (Frankel et al., 2002). Once internalised, the HBEGF-precursor receptor / DT complex follows the degradative pathway described above (Fig. 23). For translocation of the A domain into the cytosol, maturation of DT is required by cleavage between R193 and S194 of the continuous polypeptide chain. This is performed by the ubiquitous membrane-bound endoprotease furin that located at the cell surface and within the endosomal system. The mature DT consists of the domain A (1-193) linked to the domains RT (194-535) by a disulphide bond between the cysteine residues C186 and C201 (Fig. 24) (Frankel et al., 2002; Gordon et al., 1995; Moskaug et al., 1989; Thomas et al., 2002). Acidification of the endosomal environment (Faundez and Hartzell, 2004) at a pH≤ 6 (Finkelstein et al., 2000) produces a conformational change in the T-domain (Fig. 25) which inserts itself into the endosomal membrane and the A-domain is translocated into the cytosol where the disulphide bond between C186 and C201 is reduced. The A domain goes into a molten-globule state which interacts with the T-domain and the translocation requires cytosolic factors (Ratts et al., 2003). The A fragment exerts its toxicity by ADP-ribosylating elongation factor 2 (EF2) at a post-translationaly modified histidine residue (diphtamide), inactivating protein synthesis (Collier, 2001). HBEGF precursor is expressed on macrophages, smooth muscle and vascular endothelial cells (Naglich et al., 1992).

We first wanted to validate a model for the targeted cytosolic translocation of therapeutic peptides into cervical cancer cells using DT. This is sizeably different to the simple targeting of the DT A-domain toxicity as has been widely validated by others (Frankel et al., 2002). Our original idea was to replace the R-domain with the α-peptide and the toxic A-domain with the peptide tag myc, thus avoiding the toxicity of the A-domain. However, only N-terminal extensions of the A-domain have been confirmed (Stenmark et al., 1991). In order to validate the proficiency of α-peptide mediated internalisation of DT, we devised preliminary experiments in which the A-domain was preserved. Use of wild type DT is considered hazardous (Collier, 2001), so for health and safety considerations we employed the mutant Glu148Ser (Barbieri and Collier, 1987) which has 800-1000 fold reduced activity and can be handled using good laboratory procedures (Sjur Olsnes, personal communication). We genetically constructed a modified DT Glu148Ser protein where the N-terminus is extended

by the amino acids of the myc epitope (EQKLISEQDL) against which antibodies have been generated and the C-terminal amino acids encompassing the R-domain where replaced with the amino acids encoding the α-peptide of Article A. We describe that the α-peptide successfully targets the modified DT protein to the endosome-lysosome system however, we were unable to demonstrated cytosolic release of the A-domain.

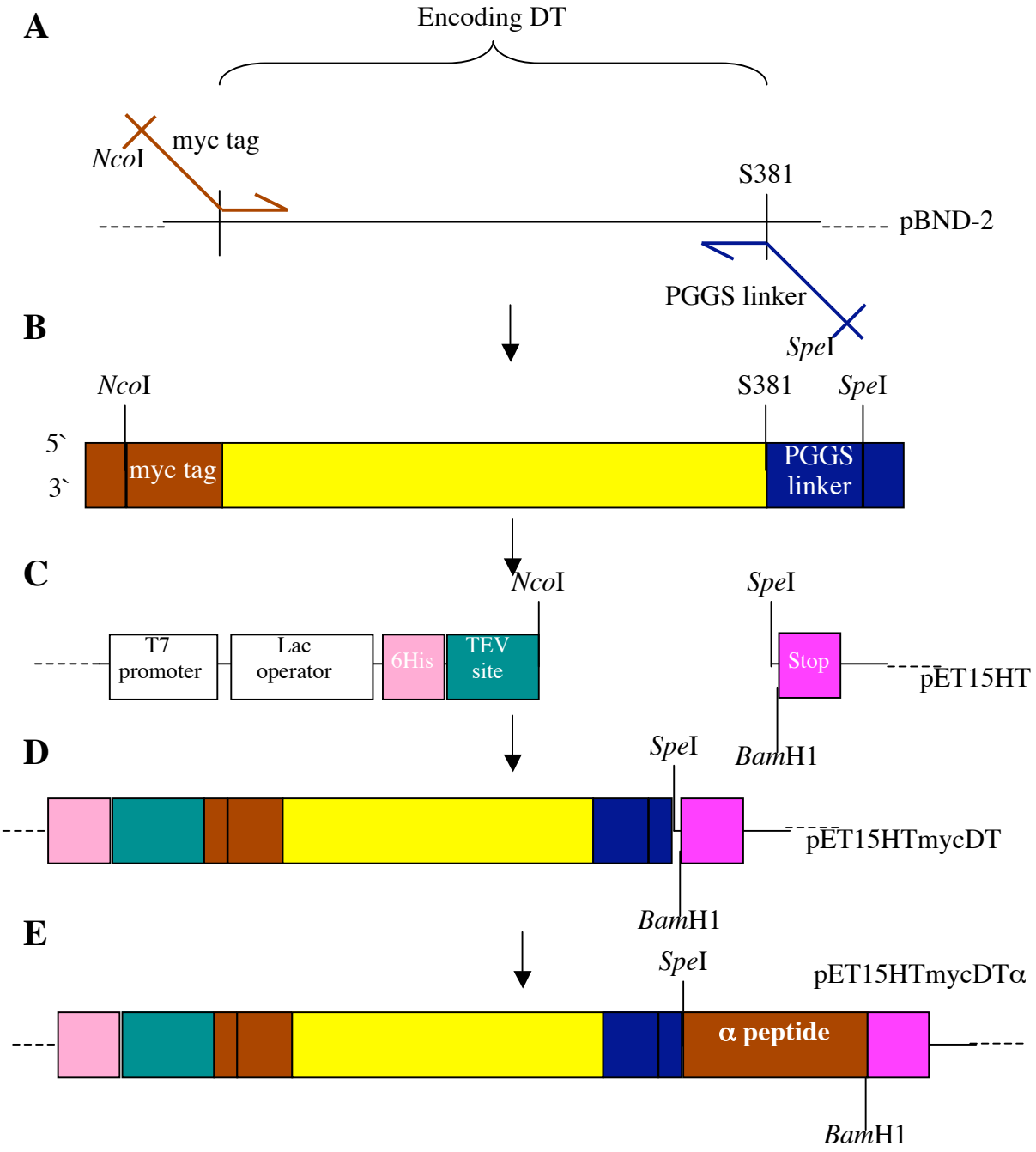

Figure 26.
Cloning of diphtheria toxin.
(A) Using the pBND-2 vector (McGill *et al.* 1990) as template DNA, the sequence encoding the first 381 amino acids of diphtheria toxin (Glu148Ser) was amplified by PCR using oligonucleotides. This generated a DNA fragment with a sequence encoding the 381 amino acids of DT with the myc peptide epitope as an N-terminal extension. (B) This DNA fragment was flanked by *Nco*I and *Spe*I restriction enzyme sites that were used to insert this fragment into the pET15HT expression vector (C) creating the expression vector pET15HTmycDT (D). A DNA fragment with cohesive ends corresponding to the restriction enzyme sites *Spe*I and *Bam*HI and encoding the α1 peptide was generated by hybridising two synthetic oligonucleotides. This fragment was inserted between the *Spe*I and *Bam*HI restriction enzyme sites of pET15HTmycDT (E).

8.2 Materials and Methods

8.2.1 Preparation of bacterial expression vectors

Using the pBND-2 vector (McGill et al., 1989) as template DNA for the sequence encoding E148S DT and oligonucleotides, a polymerase chain reaction (PCR) was performed using the Expand DNA polymerase kit (Roche) and the iCycler (Biorad) following the manufacturer's instructions. The purified PCR product was inserted between the unique restriction enzyme sites *Nco*I and *Spe*I of the pET15HT vector (Orfanoudakis, unpublished results) to give pET15HTmycDTat encoding, from 5' to 3', the myc peptide tag, the A-domain, the T-domain and finally a stop codon (Fig. 26). A DNA fragment encoding the α-peptide was generated with cohesive ends by hybridisation of two synthetic oligonucleotides and inserted between the *Bam*HI and *Spe*I restriction enzyme sites of pET15HTmycDTat to give pET15HTmycDTatα. All restriction enzymes and the calf intestine phosphatase used were from New England Biolabs, T4 Kinase was from Amersham Biosciences, purification of DNA fragments was performed using GFX kit (Amersham Biosciences) and the ligation of DNA was performed using the DNA Ligation System (Amersham Biosciences).

8.2.2 Bacterial expression and purification of recombinant diphtheria toxin

BL21/DE3 *E. Coli* bacteria (Novagen) transformed by the vectors pET15HTmycDTat or mycDTatα were incubated in LB in the presence of 50 μM ampiciline, 34 μM chloramphenicol and 0.2% glucose at 37 °C with shaking until OD_{600nm} reached 0.5. Expression was induced by addition of 500 μM IPTG at 28°C for 6 h. Bacteria were collected by centrifugation (2500g, 30 min), resuspended in 20 mM phosphate buffer pH 7.4, 30 mM imidazole in presence of EDTA-free antiprotease cocktail Complete (Roche). The bacterial suspension was then sonicated (Vibra-cell, Bioblock Scientific) and filtered. The clarified lysate was passed onto a 5 mL Hi-Trap Ni^{2+} chelate column (Amersham Biosciences). The column was washed with 30 mM imidazole and the specifically bound proteins eluted with 300 mM imidazole in the same buffer. Imidazole from pooled fractions containing the protein was removed using a PD-10 column (Amersham Biosciences) and the buffer was replaced by

PBS. The proteins were then digested by recombinant 6His-TEV protease for 12 h at 16 °C and passed on a 5 mL Hi-Trap Ni^{2+} chelate column. The flow through containing the protein was desalted using a PD-10 column and concentrated by centrifugation (Amicon Centricon Centrifugal Filter device, Millipore). To remove any protein aggregates, gel filtration was performed using a Superdex 200 gel filtration column (Amersham Biosciences). Following concentration of the fraction containing the monomeric protein. The concentration was determined by absorption at 280 nm.

8.2.3 Immunofluorescence microscopy

SiHa cells were grown to subconfluence in 8 chamber Lab-Tek permanox slides (Nunc) in DMEM supplemented with 10% fœtal calf serum (FCS), 50 μg/mL penicillin and streptomycin (Invitrogen). Following pre-incubation (where indicated) with 50 μM monensin (Sigma-Aldrich) for 30 min, the cells were incubated with the purified proteins (final concentration 100 μg/mL) mycDTat and mycDTatα diluted in 500 μL DMEM in the presence or absence of 50 μM monensin for 8 hours (all incubations at 37°C, 5% CO2). All subsequent steps were performed at room temperature. Cells were washed three times with PBS Ca^{2+}/Mg^{2+}, fixed by incubation with 4% w/v paraformaldehyde in PBS for 15 min, washed with PBS and finally permeabilised by incubation with 0.1% v/v triton X-100 for 10 min. They were then probed by incubation for 1 hour with mouse 9E10 anti-myc antibody and an anti-mouse TRITC antibody, respectively diluted 1:2000 and 1:1000 in DMEM 10% FCS. Cells were visualised by fluorescence microscopy using the Axio vert Fluorescence microscope (Zeiss), magnification was 400 times.

8.2.4 Maturation of diphtheria toxin by furin proteolysis

50 μg of DT protein and 2 units of soluble furin (2U/μL) (Sigma) were incubated for 1 h at 30 °C in 40 μL buffer (100 mM Hepes, 1mM CaCl2). 5 μL of this was boiled in SDS Loading Buffer 2% w/v SDS, 1% v/v glycerol, 0.5% v/v β-mercaptoethanol 0.1% w/v bromophenol blue 50 mM Tris HCl pH 6.8 in the presence or absence of β-mercapto ethanol, separated by 12% SDS-PAGE and either stained by coomassie blue or subjected to Western blotting. Using a semi-dry Western blotting apparatus (Owl), proteins were transferred to the nitrocellulose membrane (Schleicher and Schuell Bioscience). Following blocking by

incubation with 5% w/v non-fat milk powder in PBS, the membrane was then probed with the anti-Myc 9E10 mouse monoclonal antibody (Santa Cruz) diluted 1:5000 in TBS 0.1% NP-40 (Sigma-Aldrich) and a rabbit anti-mouse antibody conjugated to Horse Raddish Peroxidase (HRP) diluted 1:4000 in the same buffer. Revelation was performed by incubation with the HRP substrate TMB (Promega).

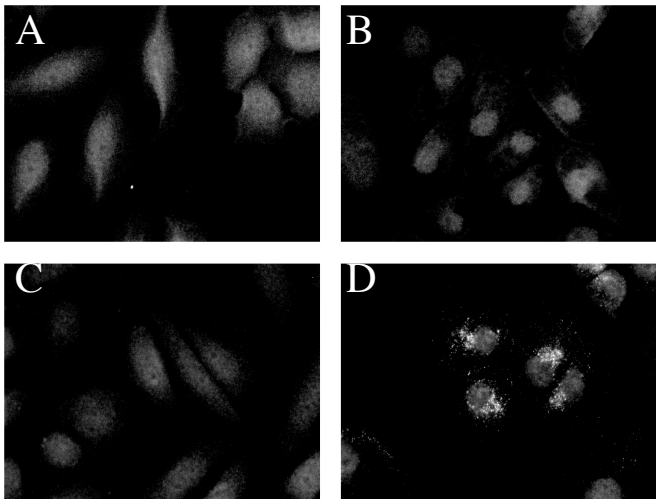

Figure 27.
mycDTatα is internalised into SiHa cells.
SiHa cells were incubated with 100 μg/mL recombinant mycDTat (A and B) or mycDTatα (C and D) in the absence (A and C) or presence (C and D) of 50 μM monensin for 8h at 37°C.

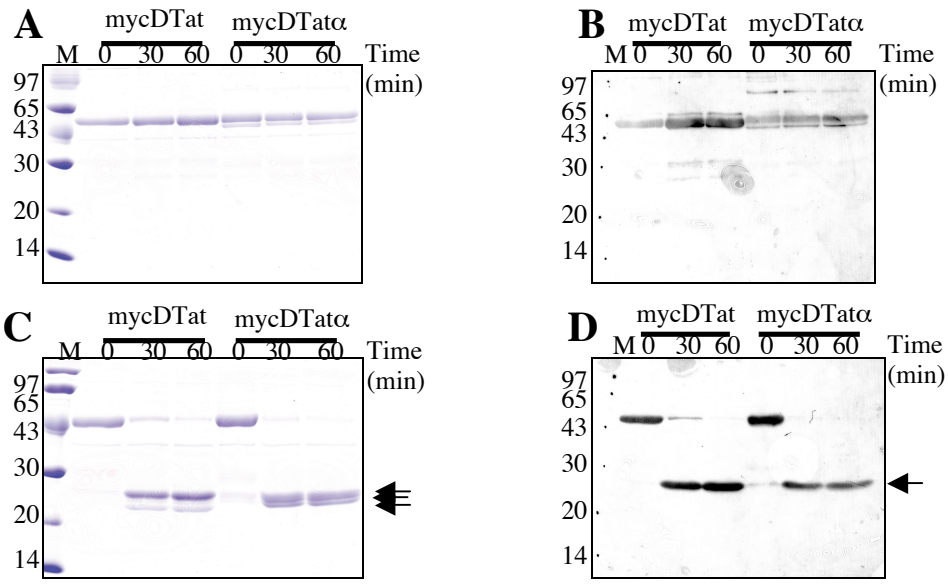

Figure 28.
Proteolysis of recombinant DTat and DTatα proteins by furin protease.
50 μg of recombinant purified DTat was incubated with 2 units of furin were incubated in the presence of calcium for upto 1h at 30°C. Proteolysis was monitored by taking aliquots and separation by SDS-PAGE under non-reducing (A and B) or reducing conditions (C and D). The gels were then either stained by coomassie blue (A and C) or subjected to Western blotting, probing for the myc epitope tag (B and D). Arrows in C indicate, in decreasing order of molecular weight, the position of mycA-domain fusion protein, T-domain α-peptide fusion and T-domain alone. Arrow in D indicates the position of the immuno-reactive mycA-domain. Molecular weight markers, M, are indicated.

8.3 Results

SiHa cells were incubated with 100 μg/mL mycDTat or mycDTatα for 8h in the presence or absence of monensin (50 μM) and then visualised by immunofluorescent microscopy (Fig. 27). A perinuclear signal was generated only in SiHa cells incubated with the DT construct fused with the α-peptide (mycDTatα) (Fig. 27). One explanation could be that this may be due to ineffective maturation of the DT proteins by integral furin of the SiHa cells. To investigate this, maturation by furin proteolysis was studied *in vitro* (Fig. 28). After 1h, the DTat was completely nicked by the 2 units of furin. In non-reducing conditions the two polypeptides of the nicked protein were maintained (A and B) whereas under reducing conditions these became separated. This strongly suggests that the furin proteolysis correctly matures the DT and that the resulting polypeptides are only maintained by disulphide linkage. However, when SiHa cells were incubated with the matured DTat (100 μg/mL) the results generated were similar to those obtained with immature DT (data not shown).

8.4 Discussion

The data presented in Fig. 27 demonstrates clearly that by replacing the R domain of DT by the α-peptide directs its internalisation into the SiHa cells (compare Fig. 27 b and d). However, it seems that a great majority of the myc-tagged A-domain is not translocated to the cytoplasm of the targeted cells before it is degraded in the lysosome. Indeed, immunofluorescence signal can only be seen in cells incubated with mycDTatα in conjunction with monensin. Monensin, an ionophore, destabilises endosomal acidification and, in doing so, protects internalised material from lysosomal degradation. One possible explanation is that the DT is not proteolyticaly matured. Indeed, endogenous furin protease, although ubiquitous, may be repressed in SiHa cells or be present in insufficient quantities to process the amounts of recombinant DT used here. To address this point, purified mycDTat and mycDTatα were subjected to furin proteolysis (Fig. 28). However, immunofluorescence images of SiHa cells incubated with such proteins gave similar results to that seen for non-matured proteins, suggesting that this is not the reason for a lack of domain A-translocation.

The reasons for the apparent failure to induce cytosolic translocation of tagged A-domain are explored below.

For insertion of the T-domain into the lipid bilayer of the endosomal membrane, a minimum distance is required as the R-domain remains bound to its receptor (Takahashi et al., 2001) which may be prevented when the R-domain is replaced by the α-peptide. However, Substance P, an 11 amino acid neuropeptide has been successfully used to replace the R-domain (Benoliel et al., 1999), hence, this may be a problematic feature of the receptor targeted by the α-peptide. Further down the pathway taken by internalised DT, A-domain translocation is inherent dependant on reduction of the disulphide bond covalently linking it to the membrane-spanning T domain (Papini et al., 1993) and on cytosolic factors (Ratts et al., 2003), an explanation could therefore lie in a deficiency of the target cells to mediate this. However, a more striking fact is that only one molecule of wild type A-domain in the cytosol of the cell is sufficient to inhibit protein synthesis (Yamaizumi et al., 1978). This points to a system which natural selection resolved poorly efficient translocation with highly efficient toxicity of the domain A. Yet Papini *et al*. estimate that over 1000 molecules of the A-domain are translocated per cell in cultured Vero cells incubated with micromolar concentrations of wild type DT (Papini et al., 1993).

This brings into question the method we chose to follow the translocation of domain A, that is to say immunofluorescence microscopy. In the literature, practically all data obtained for the A domain translocation has been obtained by using *in vitro* radio-labelled proteins or by the measurement of the incorporation of radiolabelled amino acids into synthesised proteins. In order to obtain a signal with the immunofluorescent microscopy, we incubated the cells with the purified protein products at a concentration of upto 2 μM. This is substantially higher than the concentrations used in the literature by 1000 fold (Barbieri and Collier, 1987; Papini et al., 1993). Thus, immunofluorescence microscopy may not be sufficiently sensitive to resolve the translocated domain A from the background noise. Furthermore, this relatively high concentration of DT may have an adverse effect on the translocation of the domain A into the cytosol. At pH 5 oligomeric, precipitated forms of DT are found (Bell et al., 1997; Steere and Eisenberg, 2000), therefore, at high concentrations and at the endosomal pH, an insoluble form of the DT could inhibit the correct translocation function possessed by a less concentrated DT. Additionally, the myc peptide fused to the N-

terminus of the A-domain may itself hinder its free translocation. Indeed, the translocation procedure is not fully understood but a model has been postulated. Hammond and co-workers propose a model in which, at endosomal pH, the A domain aquires a molten globule state and the T domain "pore" acts as a chaperone for its step-wise translocation (Fig. 25) (Hammond et al., 2002). Although peptides fused to the N-terminus of the A-domain have previously been described and do not impede translocation (Hammond et al., 2002; Stenmark et al., 1991), the myc peptide sequence has not been previously studied and therefore may hinder A-domain translocation.

8.5 Conclusion

We have directed cellular binding and internalisation of DT SiHa cells by replacing its receptor-binding domain by the α-peptide that shows specificity to cultured HPV-transformed cells. However, translocation of the A-domain to the cytosol of these cells cannot be demonstrated. For reasons discussed in great detail above, this is due to either the protein construction, the targeted cells, the method employed to measure the translocation or a convergence of these. I believe that it is more likely that the use of immunofluorescence microscopy is the principle limiting-factor for this. I propose therefore, that further experiments using radiolabelled DT should be performed in order to investigate translocation of A domain directed to cells by the α-peptide. Indeed, instead of using bacterially expressed protein we should first investigate whether the α-peptide allows A domain translocation. Stenmark and co-workers used a reticulocyte lysate system to produce radiolabelled proteins which they bound to cells and nicked using trypsin before briefly exposing them to acidic pH. Mimicking the endosomal pH in this way induced the cytosolic translocation of the A domain (Stenmark et al., 1991). Using this system, the quantities of protein produced are relatively small therefore, the health and safety considerations would allow us to use whole Glu148Ser DT. This would provide us with a positive control that was lacking in the experiments performed previously. This would also allow us to investigate the other internalising peptides described in Article A.

9. Manuscript B (to be submitted)

Distinct regions within p53 core domain control E6-dependent and E6-independent p53 cellular degradation processes.

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9.1 Abstract

The best studied molecular property of high-risk HPV E6s is their ability to stimulate cellular degradation of the tumour suppresser p53 via formation of a trimeric complex comprising of E6, p53 and E6-AP. HPV16 E6 (16E6) binds at least two distinct sites on p53. One site, situated in the p53 C-terminal region, is recognised by both high and low-risk HPV E6s. The second site is situated within the core domain of p53 and is only recognised by high-risk HPV E6s, associated with E6-AP.

Using recombinant purified 16E6 we screened a phage-peptide display library presenting 12-mer peptide sequences. One subset of the sequences isolated presented significant homology with a short stretch of amino acids ^{261}SGNLLG^{266} within the p53 core domain. We demonstrated that a peptide corresponding to this region bound 16E6 and a full-length p53 protein bearing a single point mutation (L265A) within this region displayed decreased affinity for 16E6. This full-length p53 mutant protein exhibited resistance to 16E6-mediated degradation *in vitro* and *in vivo*. Interestingly, when transiently expressed in Saos-2 cells, the p53 L265A mutant was rapidly degraded. Further site-directed mutation of a second linear stretch of the p53 core domain completely abolished 16E6-dependent and independent degradation of p53. On the basis of these data, we propose that a conformational change within the p53 core domain, absolutely required for degradation for binding of E6/E6-AP complex and p53 degradation.

9.2 Introduction

High-risk HPV E6 protein has many cellular partners and its role in the immortalization of cells infected by high-risk HPV types is fundamental. In order to increase the understanding of these interactions and also to destabilise these interactions, peptide ligands of the E6 protein have been isolated. This strategy has enabled the identification of the "charged leucine" motif used by several cellular partners of E6 (Elston et al., 1998) and peptide ligands that induce apoptosis in human cells transformed by HPV (Butz et al., 2000).

The two studies performed by Elston *et al*. and Butz *et al*. used yeast-two-hybrid technology to screen peptide libraries (Butz et al., 2000; Elston et al., 1998). Phage-peptide display is an alternative technique that has been previously used to screen peptide libraries on purified proteins. This approach has allowed the identification of interacting surfaces. For example, the MDM2-binding domain of p53 has been successfully characterised by phage display using linear peptide libraries on purified MDM2 protein (Bottger et al., 1997; Bottger et al., 1996). However, full-length E6 protein is notoriously difficult to purify and work performed in our laboratory has addressed this problem by the characterisation of a soluble, mono-disperse and biologically functional mutant of 16E6. In this E6 mutant, the 6 cysteine residues not conserved in the E6 sequences of the different HPV types are replaced by serines (16E6 (6S/6C)) (Nomine et al., 2001a; Nomine et al., 2001b). In this study, we used a purified sample of this mutant to screen a phage display library of linear 12-mer peptides. Initially, the biopannig procedure was performed on recombinant purified 16E6 (6S/6C) protein immobilised on the wells of a plastic micro-well plate. This experiment generated a first collection of peptides complemented later by performing the same biopanning on 16E6 (6S/6C) using different immobilisation techniques. The different protocols led to the isolation of 165 individual peptide sequences that were classified into 12 groups following sequence homology. One group of peptides was found to be homologous to the sequence spanning the amino acids ^{261}SGNLLG^{266} of p53 which may represent the second binding domain of high-risk E6 to p53. Indeed, it has been found that high and low-risk E6 protein bind the C-terminal region spanning amino acids 376 to 384 of p53 and, when associated with E6-AP, only high-risk E6 protein binds a second domain within the p53 core domain that is required for degradation (Li and Coffino, 1996a). Interestingly, further investigation of MDM2 has revealed that this same region is also important for its interaction with p53. MDM2 bound to RNA has an altered conformation (Burch et al., 2000) and biopanning experiments on this

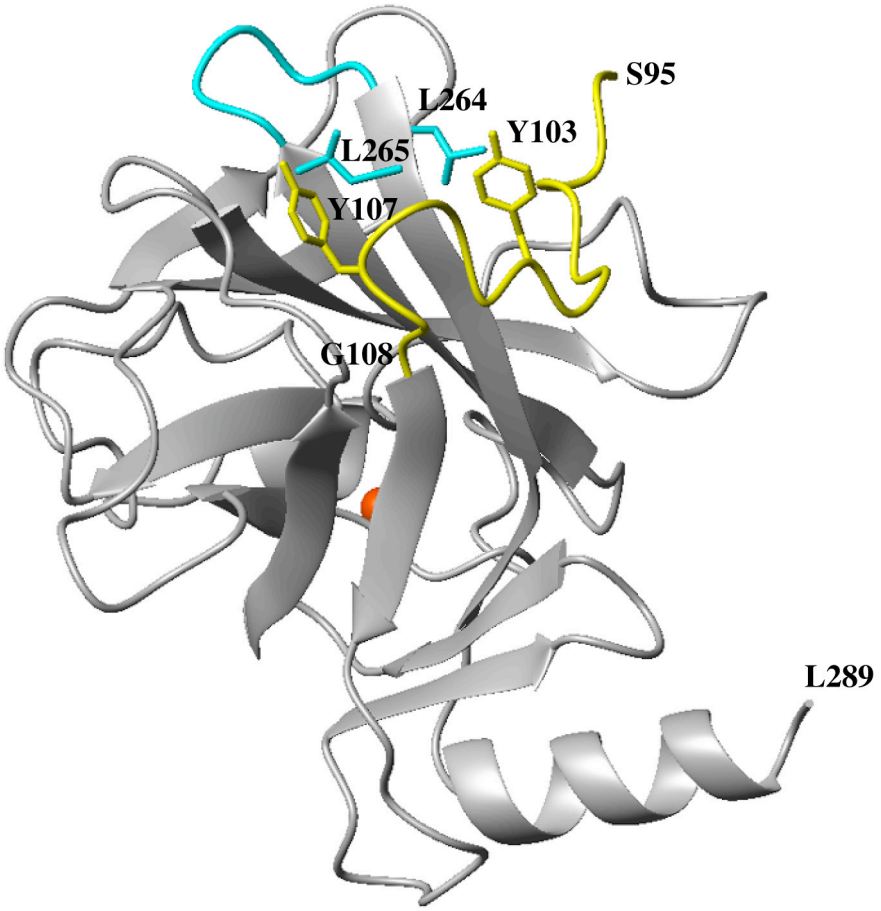

Figure 1.
Representation of the p53 core domain tertiary structure.
The core domain encompasses the DNA-binding domain of p53 and is the only part of p53 for which the tertiary structure has been defined. The region spanning the amino acids 261 to 265 (blue) are on the opposite side to the DNA-binding domain (situated at the base of this representation). Leucine 264 and L265 interact closely with the two tyrosines 103 and 107 (yellow) which are part of the N-terminal arm of the core domain that spans the amino acids 95 to 108.

form of MDM2 isolated peptides with homology to the region spanning ^{261}SGNLLGRNSF^{270} of p53 core domain (Shimizu et al., 2002). As can be seen in the representation of the 3D structure of the p53 core domain (Fig. 1), the amino acids ^{261}SGNLLG^{266} make direct contact with a region previously identified as being important for p53 degradation. Indeed, the amino acids 101 to 131 have been found to be important in ubiquitin-independant p53 lability (Li and Coffino, 1996b) and amino acids 92 to 112 important for both E6 and MDM2-mediated ubiquitin-dependent degradation (Gu et al., 2001).

To explore the pertinence of the phage display results, we first demonstrated the binding of 16E6 to the region ^{261}SGNLLG^{266}. Subsequently, we performed point mutation analyses on this region and demonstrated that the amino acid L265 is important for 16E6-mediated degradation of p53 and for 16E6 binding to p53. Interestingly, mutation of this residue caused rapid degradation of the p53 protein when expressed in the p53 null cell line Saos-2 but not in H1299 cells, another p53-null cell line. This was further investigated by mutation of the residues spanning the region 99 to 107 which are within the region previously described as important for p53 degradation and in contact with the region 261 to 265. The mutation of these residues resulted in a p53 protein highly stable in the Saos-2 cells and resistant to 16E6-mediated degradation in reticulocyte lysates and both Saos-2 and H1299 cell lines suggesting that 26S proteasome-mediated degradation of p53 is determined by this region.

9.3 Materials and Methods

Biopanning

The Ph.D.–12 phage-peptide library (New England Biolabs) was used for biopanning experiments on recombinant 16E6 protein. This M13 phage library uses the N-terminus of M13 minor coat protein pIII to display random peptide sequences of 12 amino acids. An individual well of a PVC micro-well plate was coated with 5 μg/mL of purified recombinant 16E6(6C/6S) (Nomine et al., 2001a; Nomine et al., 2001b) in PBS (130 mM NaCl, 7mM $Na_2HPO_4 \cdot 12H_2O$, 3 mM NaH_2PO_4, pH 7.5) and incubated at 4°C overnight with agitation in a humidified chamber. Unbound protein solution was removed, the wells washed twice with PBS and the coated well was then incubated with 200 μL blocking buffer (100 mM $NaHCO_3$, 5 mg/mL BSA, 0.02% NaN_3, pH 8.6) for 1h under the same conditions. To the coated well

Table 1. Construction of p53 expression vectors.

NAME	SEQUENCE	
pcDNA-Wt	^{99}SQKTYQGSY^{107}	^{261}SGNLLG^{266}
pcDNA-S261A	^{99}SQKTYQGSY^{107}	^{261}**A**GNLLG^{266}
pcDNA-N263A	^{99}SQKTYQGSY^{107}	^{261}SG**A**LLG^{266}
pcDNA-L264A	^{99}SQKTYQGSY^{107}	^{261}SGN**A**LG^{266}
pcDNA-L265A	^{99}SQKTYQGSY^{107}	^{261}SGNL**A**G^{266}
pcDNA-99-107	^{99}**GAGAGAGAG**^{107}	^{261}SGNLLG^{266}
pcDNA-99-107 L265A	^{99}**GAGAGAGAG**^{107}	^{261}SGNL**A**G^{266}

Sequences of the two stretches subjected to point mutagenesis are shown. Mutated amino acids are indicated as bold blue characters.

was added 4 × 10^{10} cfu of the phage library, diluted in 100 µL blocking buffer, 0.1 % v/v Tween-20 (Sigma-Aldrich) and incubated for 1h at room temperature in a humidified chamber with agitation. The phage solution was removed and the well washed 10 times with TBS (50 mM Tris, 150 mM NaCl, pH 7.5), 0.1% Tween-20 (TBST 0.1%) and the bound phage eluted by incubation with elution buffer (100 µL 0.2M Glycine-HCl, pH 2.2, BSA 1 mg/mL) for 10 min at room temperature. The eluate was immediately neutralised with 15 µL 1 M Tris-HCl pH 7.5. Amplification, purification and titration were performed as previously described (Choulier et al., 2001). Stringency was augmented in subsequent rounds of selection by increasing the concentration of Tween-20 to 0.5% v/v in the incubation solution. After three successive rounds of biopanning, individual clones were amplified and the amino acid sequence displayed deduced as previously described (Choulier et al., 2001).

Plasmid construction

Using standard PCR protocols and the plasmid vector pCMV-neo-bam as the DNA template (Baker et al., 1990), DNA sequences encoding the various forms of human p53 were obtained (Table 1). Using restriction enzymes (New England Biolabs) and the DNA Ligation system (Amersham Biosciences), these sequences were inserted between the unique restriction sites *Eco*RI and *Not*I of the expression vector plasmid pcDNA1-Amp. The plasmid encoding wild-type p53 was named pcDNA-Wt and those encoding point mutations of the amino acid sequence in the region ^{261}SGNLLG^{266} were named pcDNA-S261A, -N263A, -L264A and -L265A respectively. The plasmid encoding the mutation of Wt p53 sequence ^{99}SQKTYQGSY^{107} to ^{99}GAGAGAGAG^{107} was named pcDNAp53-99-107, when combined with the L265A mutant, generated the plasmid pcDNAp53-99-107 L265A. The expression vector pcDNAE6K2 encoding the HPV16 E6 protein has previously been described by (Giovane et al., 1999).

GST fusion protein expression vectors were constructed using the pETM30 expression vectors kindly provided by G. Stier (EMBL, Heidelberg, http://www-db.embl.de). DNA oligomers encoding the p53 core domain peptide (^{255}TLEDSSGNLLGR^{267}) or random peptide (GSNSGNGNS) were inserted between the restriction sites *Nco*I and *Kpn*I of the pETM30, a modified pET24d vector containing a N-terminal His6-GST tag and a TEV protease cleavage site. This gave rise to the vectors pETM30p53pep, pETM30random pep.

MBP fusion protein expression vectors were constructed using the pETM-vectors, again, kindly provided by G. Stier. The sequence encoding the 16E6 mutant 6C/6S (Nomine et al., 2001a; Nomine et al., 2001b) was inserted between the restriction sites *Nco*I and *Kpn*I of the pETM-41, a modified pET24d expression vector containing an N-terminal His6-MBP tag and a TEV protease cleavage site. This gave rise to the vector pETM-41E66C/6S. DNA oligonucleotides coding for the e6-ap peptide (^{393}ESSELLQELLGEER^{408}), random peptide (GSNSGNGNS), the p53 peptide (^{256}TLEDSSGNLLGR^{267}) and the "NLLG" peptide isolated by phage display (YGAHISVNLLGQ) were inserted between the restriction sites *Nco*I and *Kpn*I sites and gave rise to the vectors pETM30randpept and pETM30p53pep and pETM30NLLGpep.

In vitro degradation assay

^{35}S-Labelled proteins were obtained by *in vitro*-translation using the TnT Quick Coupled Transcription/Translation rabbit reticulocyte lysate system (Promega) following the manufactures instructions and using Promix ^{35}S-labelled Cysteine and Methionine (Promega). The following plasmids were used to generate the according protein products: pcDNA-E6K2, pcDNA-Wt, pcDNA-S261A, pcDNA-N263A, pcDNA-L264A, pcDNA-L265A, pcDNA-99-107 and pcDNA-99-107 L265A. The lysates containing translated E6 protein (25 µL) and the indicated translated p53 protein (10 µL) were incubated at 28°C in the presence of 3 mM DTT, 100 mM NaCl, 25 mM Tris-HCl, pH 7.5 in a final volume of 50 µL. At the indicated times, 5 µL aliquots of these reactions were added to SDS sample buffer. The samples were separated by 12% SDS-PAGE and the gel was incubated for 45 min in fix buffer (20% v/v ethanol, 10% v/v acetic acid) and dried before exposure to a BioMax MR film (Kodak).

Coimmunoprecipitation of p53 and E6

Immunoprecipitation was performed with ^{35}S-labelled proteins obtained as described above and using the plasmids pcDNA-E6K2, pcDNAp53-Wt and pcDNA-L265A. The indicated combination of the resulting rabbit reticulocyte lysates containing translated p53 Wt or L265A (2 μL) and/or translated E6 (10 μL) were incubated in 200 μL PBS supplemented with Complete EDTA-free antiprotease cocktail for 1h on ice. Subsequently, to this was added: 25 μL Pab1801 anti-p53 monoclonal antibody (200 μg/mL) 50 μL Protein A-Sepharose equilibrated in PBS (Amersham Biosciences) and 1 mL PBS, 0.5% v/v NP-40 (Sigma-Aldrich), 1% w/v BSA supplemented with Complete EDTA-free antiprotease cocktail. After incubation overnight at 4°C on a wheel, the Protein A-sepharose was collected by centrifugation (2,000 rpm) and washed with PBS, 0.5% v/v NP-40 twice in the presence of 1% w/v BSA and then twice without BSA. The proteins were eluted with 20 μL SDS sample buffer and subjected to 15% SDS-PAGE.

Pull-down and hold-up protein interaction assays

Hold-up

Hold-up assays were performed using bacterially expressed proteins from the pETM-30 vectors described above encoding the fusion proteins MBP-random and p53 peptide along with MBP-E6 6C/6S (Nomine et al., 2001a; Nomine et al., 2001b). pETM expression constructs were electroporated into BL21 DE3 *E. coli* cells. Expression cultures were grown at 37 °C until $OD_{(600\ nm)}$ = 0.6 in LB, 15 μg/mL kanamycin and induced at 27 °C with 0.5 mM IPTG for 4-6h. Expression cultures of E6 6C/6S were adjusted to 100 μM $ZnSO_4$ before induction. Induction of GST-peptide expression was performed for 2h at 37 °C. Expression pellets were sonicated in buffer A (Tris 50 mM, NaCl 400 mM, DTT 2 mM, pH 6.8) containing 5% glycerol, 1 μg/mL DNase I, 1 μg/mL RNase I, and anti-protease cocktail (EDTA-free) (Roche), cleared by centrifugation at 18,000 *g* at 4 °C for 30 min and filtered (Millipore 0.22 μm). MBP-E6 extracts were loaded on amylose resin column (New England Biolabs) pre-equilibrated with buffer A. Protein was eluted with buffer A supplemented with 10 mM maltose, ultracentrifugated overnight at 160,000 *g* and 4 °C in a SW41 rotor (Beckman) and gel filtrated on a Hiload 16/60 Superdex 75 column (Amersham Biosciences) equilibrated in

buffer A, raising pure monodisperse MBP-E6. To obtain non-fused E6 protein, the purified MBP-E6 was further cleaved with recombinant TEV protease (Nomine et al., 2003). The digestion product was subjected to a second gel filtration raising pure monomeric E6. Hold-up assays were performed using affinity resins pre-equilibrated in buffer B (50 mM Tris, 200 mM NaCl, 1mM DTT). 25 mL pellets of bacterial expression of ligand fusion protein (MBP-random or p53 peptide) were sonicated at 4 °C in 1 mL buffer B containing 1 μg/mL DNase I, 1 μg/mL RNase I, and anti-protease cocktail, and centrifuged at 12,000 *g* for 15 min. The resulting supernatants ("crude expression lysates") were kept on ice. Ligand lysates were batch-incubated at 4 °C for 30 min in the presence of 100-300 μL maltoheptose agarose (Sigma) for MBP-ligands. Beads were then washed once for 10 min in 15 mL of buffer B supplemented with anti-protease cocktail, constituted the stock of resin saturated with a 50-100 μM concentration of pure ligand. For each interaction experiment, we prepared two 500 μL Eppendorf tubes containing 10 μL (bed volume) of ligand-saturated resin each. Both tubes were supplemented with 30 μL of buffer B containing either 5-10 μM of pure "analyte" E6 protein. Ligand-analyte interaction was finally achieved via 15 min incubation on ice, at the end of each minute this was mixed by gentle vortexing, avoiding spreading resin on the tubes sides. For each pair of samples, one tube (labelled +M) was adjusted to 10 mM of competitor molecule (maltose), by adding 4 μl of 110 mM (11x) competitor stock solution, to elute bound protein from the resin. The other tube (labelled -M) was adjusted with 6 μL of water. After another 5 min incubation at 4 °C interspersed with gentle vortexing, the liquid was extracted from the resin using a 96 well MultiScreen-HV filterplate possessing a 0.45 μm Durapore membrane (Millipore). The volumes of the extracted liquid phases were adjusted and SDS loading buffer was added before being treated as before to SDS-PAGE using a 12% acrylamide gel and Coomassie-blue staining for analysis

Pull-down

pET30 expression constructs were electroporated into BL21 DE3 *E. coli* and proteins were expressed as described for MBP fusions until obtention of lysates which were then incubated with glutathion-sepharose beads (Amersham) for 2h at 4°C. These were washed in the lysis buffer and then used for *in vitro* GST pull-down experiments. 10 μL of ^{35}S-labelled 16E6 obtained as described above were incubated with 10 μL of GST-peptide immobilised on

glutathion-sepharose beads in 300 µL of lysis buffer for 4h at 4°C. Following 4 washes, the proteins bound to glutathion were eluted by boiling in SDS-loading buffer and separated by 12% SDS-PAGE. The acrylamide gel was then incubated for 45 min in fix buffer (20% v/v ethanol, 10% v/v acetic acid) and dried before exposure to a BioMax MR film (Kodak).

Transfection assays

Saos-2 and H1299 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% v/v Fœtal Bovine serum and 50 μg/L penicillin, 50 μg/L streptomycin (Invitrogen) and maintained at 5% CO_2 at 37°C. Transfection of the indicated plasmids was performed using FuGene6 transfection reagent following the manufacture's instructions (Roche). Where indicated and at 32h post-transfection, the cells were incubated with 5 μM MG132 (Calbiochem) for the remaining 16h. For immunoprecipitation, 48h post-transfection, cells contained within 3 wells of a 6 well plate were washed twice with cold PBS and incubated 15 min with lysis buffer (1% v/v NP-40, 140 mM NaCl, 4 mM MgCl2, 20 mM Tris-HCl pH 7,5 supplemented with Complete EDTA-free antiprotease cocktail, Roche) on ice with vigorous shaking. Lysate was clarified by centrifugation (10 min, 13 krpm, 4°C) and an aliquot used to determine total protein concentration using the Bradford assay (BioRad). Volumes of the clarified lysate standardised for total protein content were then incubated with 25 μL of Pab1801-Agarose Conjugated anti p53 monoclonal antibody (Santa Cruz) equilibrated in the same buffer overnight at 4°C on a wheel. The agarose beads were collected by centrifugation (2 krpm) and washed three times with 1 mL buffer B (0.2% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5), twice with 1 mL buffer C (0.2% NP-40, 500 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 7.5) and twice with 1 mL 10 mM Tris-HCl. Proteins were eluted with SDS sample buffer subjected to 10% SDS-PAGE, blotted and probed for p53 using poly clonal rabbit anti-p53 antibodies (Santa Cruz), monoclonal mouse anti-rabbit IgG HRP-conjugated antibody (Jackson) and revealed using ECL+ chemiluminescence reagent (Amersham Biosciences). In experiments where crude cell extracts were probed for p53, the same protocol as described above was applied with the following modifications. 5×10^5 cells of were suspended by trypsination and collected in 1 mL of complete culture medium containing 10% fœtal calf serum. The cells were collected by centrifugation (1000 rpm, 5 min), rinsed with PBS and resuspended in 400 μL of lysis buffer. After sonication, the cell lysate s were cleared by centrifugation (20 min, 13,000 rpm) An aliquot was used to determine total protein concentration which determined the relative volumes (to the order of 5 μL) of the samples loaded on to SDS-PA gels as described above.

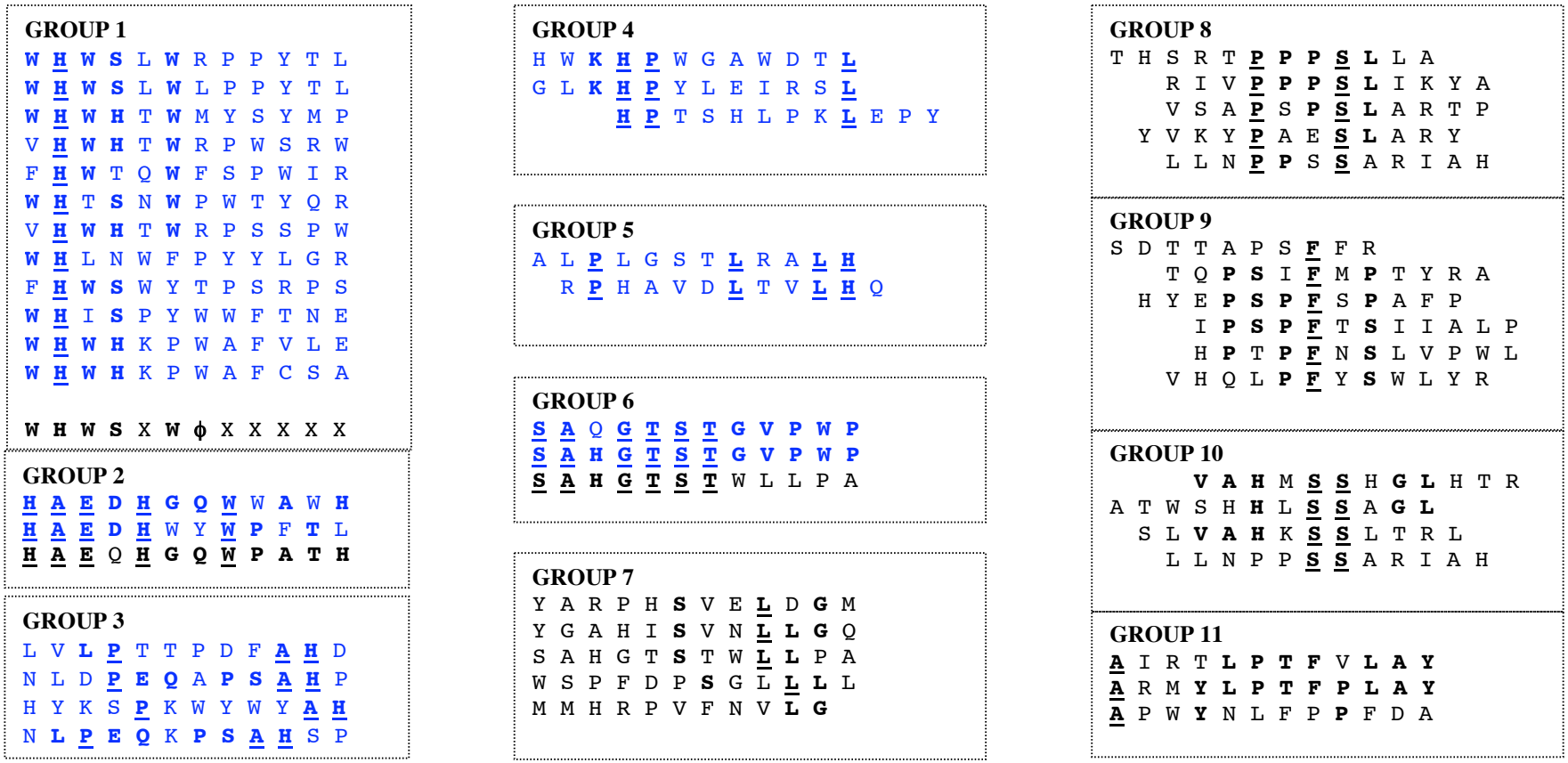

Figure 2.

Aligned peptide sequences obtained from phage display experiments.

Amino acids in bold are found to be highly conserved between the members of the same group and when underlined these are absolutely conserved. Sequences obtained from biopanning a phage display library composed of linear peptides of 12 amino acids presented on the pIII of M13 phage on plastic-immobilised recombinant 16E6 (6C/6C) protein are in blue and those obtained using sepharose-immobilised 16E6 (6C/6C) in black. Individual and groups of two sequences are not shown.

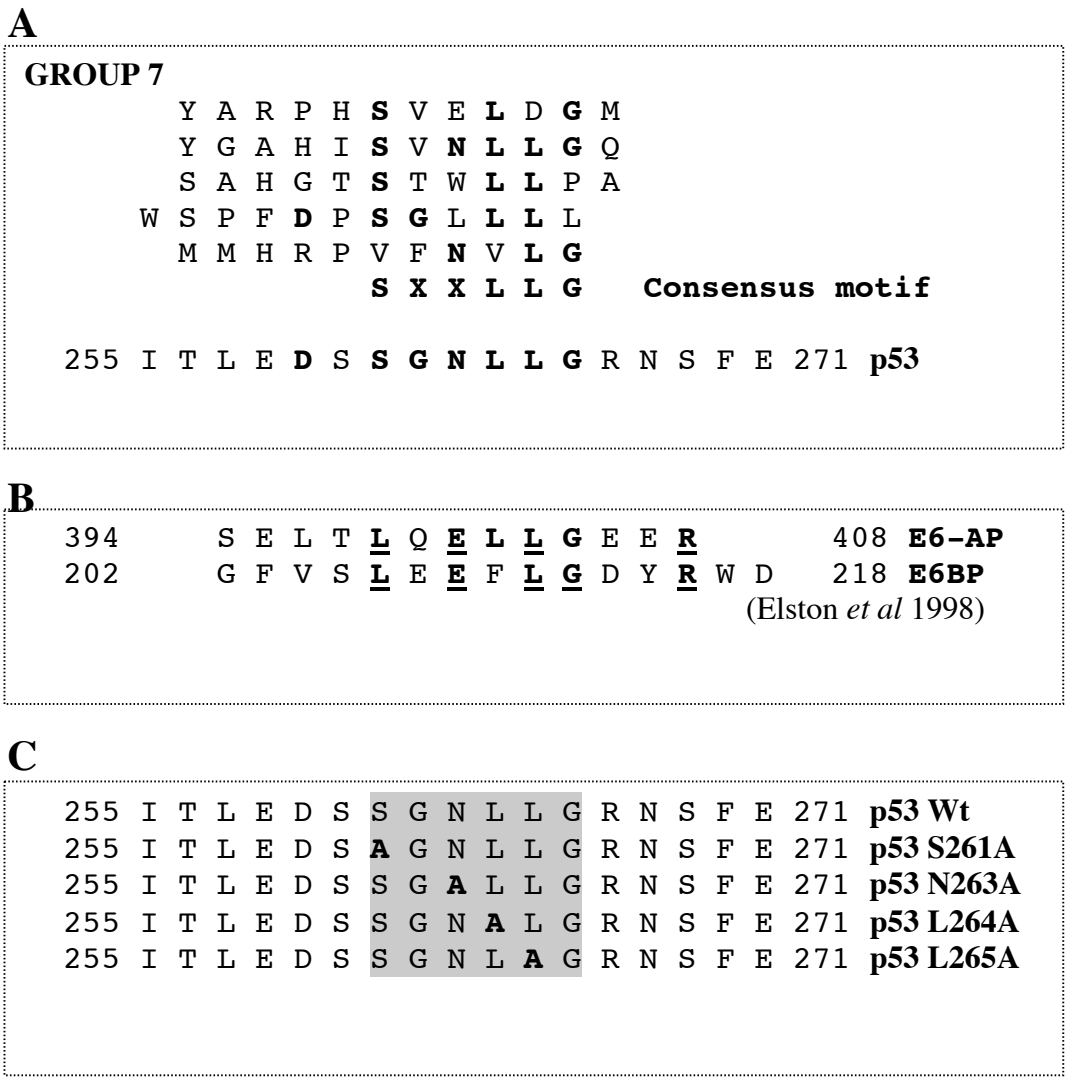

Figure 3.
Group 5 shows homology to a region in p53.
(A) It was found that the peptide sequences of Group 7 demonstrate high homology with a sequence spanning the amino acids 261 to 266 of p53 (SGNLLG). (B) Using yeast-two-hybrid technology, Elston *et al* (1998) defined 16E6-binding peptides which were found to be homologous to the minimal amino acid stretch of E6-AP required for E6 interaction along with that of E6BP. (C) Point mutagenesis of p53 protein. S261, N263, L264 and L265 were changed sequentially to alanine.

9.5 Results

Biopanning on purified HPV16 E6 protein

Recombinant HPV E6 protein is particularly difficult to purify. The work performed in our laboratory by Gilles Travé and co-workers on this subject has led to the identification of a stabilised mutant of 16E6 where non-conserved cysteines are replaced by serines, 16E6 (6C/6S). This mutant can be bacterially expressed and purified while retaining its biological activity (Nomine et al., 2001a; Nomine et al., 2001b). A sample of the purified 16E6 (6C/6S) protein was immobilised on the wells of a PVC micro-well plate and 10^{11} tu of the M13 phage library displaying random 12-mer peptides was used for biopanning. Bound phage were eluted with pH 2.2 solution and after three rounds of selection, individual clones were used to prepare phage DNA for sequencing. These experiments yielded a collection of 33 peptides that could be organised following their sequence homology into 6 groups (Fig. 2, sequences in blue). We noted a large group (Group 1) that was composed of highly hydrophobic amino acid sequences. These hydrophobic sequences could represent non-specific plastic-binding peptides, however, these form a homologous group, indicating that their selection was not random. In a second set of experiments, the same phage display library was used on 16E6 (6C/6S) immobilised on sepharose beads. E6 protein was either covalently immobilised on CNBr sepharose beads or by the complex sepharose-protein A-6F4 anti-16E6 antibody. The 6F4 antibody is raised against 16E6 and its epitope has been delineated in our laboratory to the region spanning the amino acids 8 to 15 of 16E6 (Giovane et al., 1999). The results of these experiments were combined and gave rise to 165 individual sequences that have been organised into 11 groups following sequence homology (Fig. 2, sequences in black). Only peptide sequences which could be aligned into groups composed of more than two members are shown in Fig. 2.

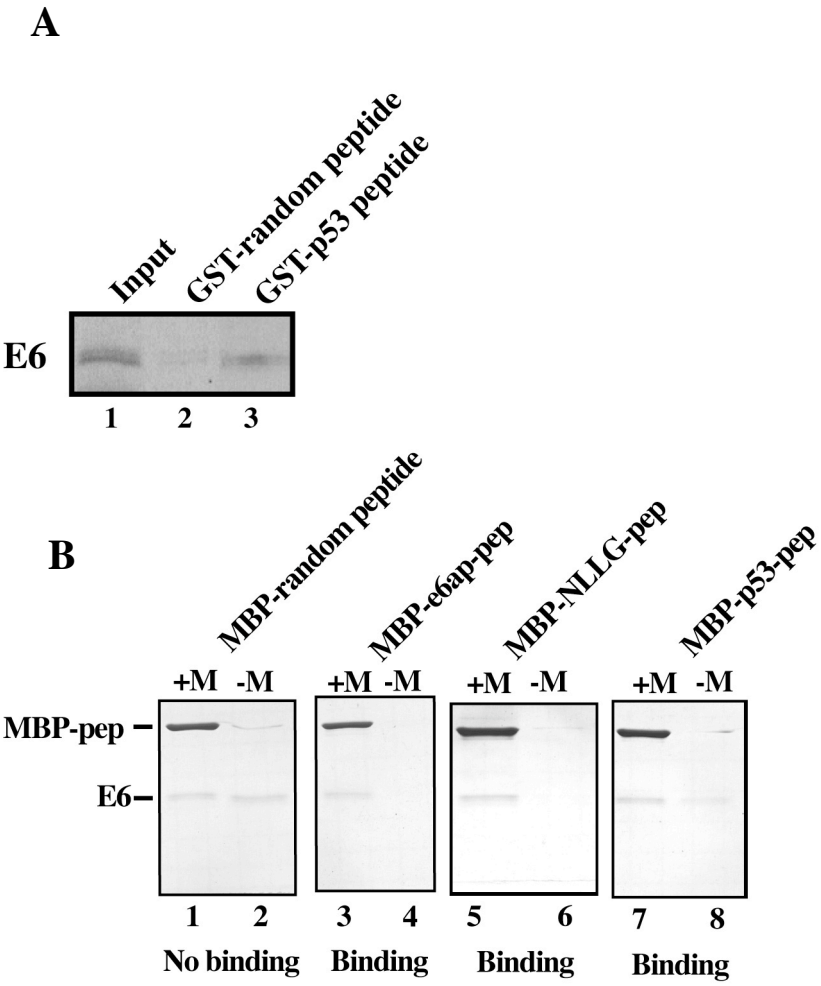

Figure 4.
Hold-up and pull-down
(A) Pull-down assay. 16E6 produced in rabbit reticulocyte lysate was incubated with GST fusion proteins consisting of either a random peptide (GSNSGNGNS) or a peptide representing the region ^{255}TLEDSSGNLLGR^{267} of p53 core domain (p53 peptide). Material bound to the GST fusion proteins was precipitated by glutathion-sepharose beads and separated by 12% SDS-PAGE before exposure to photographic film. (B) Hold-up assay. Bacterially expressed and purified 16E6 (6S/6S) and MBP-fusion proteins were incubated together for hold-up assays. The MBP was fused to the random peptide, the e6ap peptide (^{393}ESSEMMQELLGEER^{408}), the "NLLG" peptide isolated by phage display (YGAHISVNLLGQ) and the were incubated. Following incubation either the entire incubation mix (+M) or the liquid phase alone (-M) was separated by 12% SDS-PAGE and treated with coomassie-blue stain for analysis.

The Group 7 immediately drew our attention as a consensus motif for this collection of peptides, SXXLLG, previously found to be homologous to a region of p53, a significant cellular binding partner of E6 (Fig. 3 A). This consensus motif was also found to be partially homologous to the "charged leucine" motif found on proteins known to interact intracellularly with E6 such as E6-AP and E6BP (Fig. 3 B).

The region ^{261}SGNLLG^{266} of p53 is implicated in 16E6 binding

At this stage it was necessary to determine whether the region, ^{261}SGNLLG^{266}, was concerned in the interaction of 16E6 with p53. For this, we used both a classic pull-down method and the novel hold-up approach (Charbonnier *et al.*, manuscript submitted for publication) which presents the advantage of measuring the interaction at equilibrium. Using the pull-down methodology we found that 16E6 bound a peptide corresponding to the p53 core domain (^{255}TLEDSSGNLLGR^{267}) "p53-peptide" (Fig. 4 A). Using the hold-up methodology, 16E6 (6C/6S) was found to bind the e6ap peptide (^{393}ESSELLQELLGEER^{408}) and a group 7 peptide (NLLG-peptide, YGAHISVNLLGQ). Binding with the p53-peptide was occurred less well (Fig. 4 B, lanes 3 to 8). 16E6 (6C/6S) was not found to bind the random peptide (GSNSGNGNS) (Fig. 4 B, lanes 1 and 2).

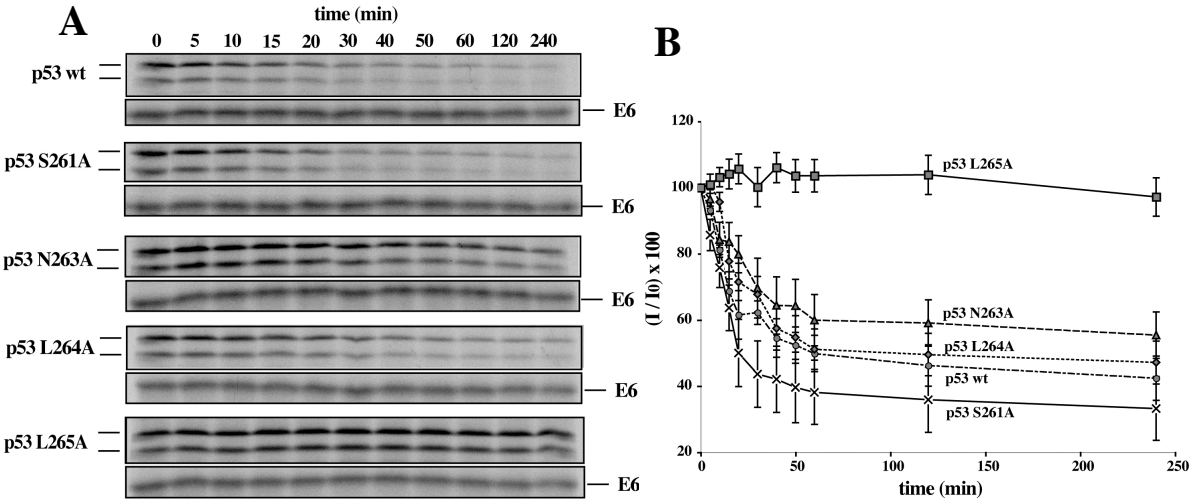

Figure 5.
16E6-mediated degradation of p53 L265A is abrogated in reticulocyte lysates..
(A) radiolabelled p53 and 16E6 proteins produced in rabbit reticulocyte lysates were incubated together at 28°C and aliquots removed at the indicated times before separation by 12% SDS-PAGE and exposition to photographic film. (B) represents the mean ± standard deviation for three independent experiments.

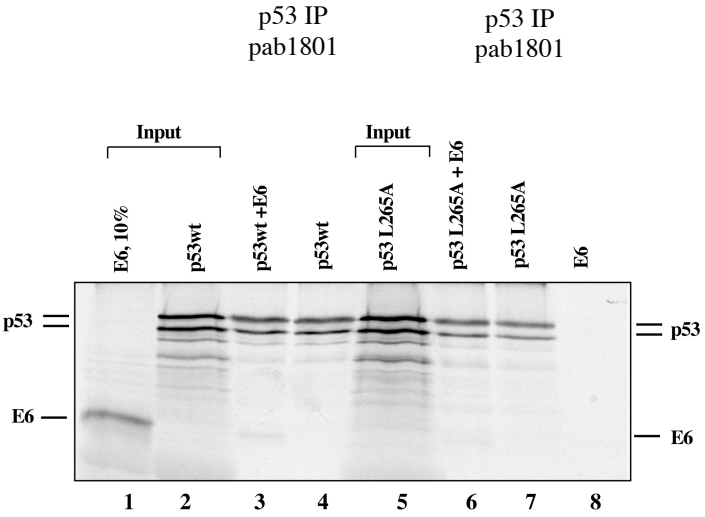

Figure 6.
L265A partially abrogates 16E6 binding to p53.
^{35}S-labelled p53 and E6 proteins produced in rabbit reticulocyte lysates were incubated together and p53 was immunoprecipitated using the Pab1801 monoclonal antibody and Protein A-sepharose beads. Coimmunoprecipitated material was separated by 15% SDS-PAGE and exposed to photographic film.

The region ^{261}SGNLLG^{266} of p53 is important for 16E6-mediated degradation

Following our observations with Hold-up and Pull-down experiments, we performed point mutagenesis on the sequence encoding whole p53 to investigate the significance of the region spanning amino acids 261 to 266 for E6-mediated degradation (Fig. 3 C). S261, N263, L264 and L265 were changed to alanine whereas the two glycine residues (G262 and G266) were not modified as it was thought that their modification could have a severe adverse effect on protein folding as glycines play an important role in structural conformation. Expression vectors encoding 16E6 and the mutant p53 proteins (p53-S261A, -N263A, -L264A and -L265A) were used to synthesise ^{35}S-labelled proteins in a rabbit reticulocyte lysate translation system. Lysates containing the according proteins were then combined in degradation assays. E6-mediated degradation of p53 requires additional proteins found in the reticulocyte lysates and is very efficient. Wt p53 along with the mutants S261A, -N263A and -L264A were promptly degraded in the presence of 16E6, but p53 L265A was found to be resistant to degradation in the same conditions (Fig. 5 A and B). Using full length p53 proteins, produced in rabbit reticulocyte lysates, it was found that the mutation L265A partially impaired coimmunoprecipitation of p53 via 16E6 (Fig. 6, compare lanes 3 and 6), indicating that whereas binding of E6 to the core of p53 was affected, the binding of E6 to the C-terminal domain of p53 remains intact (Li and Coffino, 1996a).

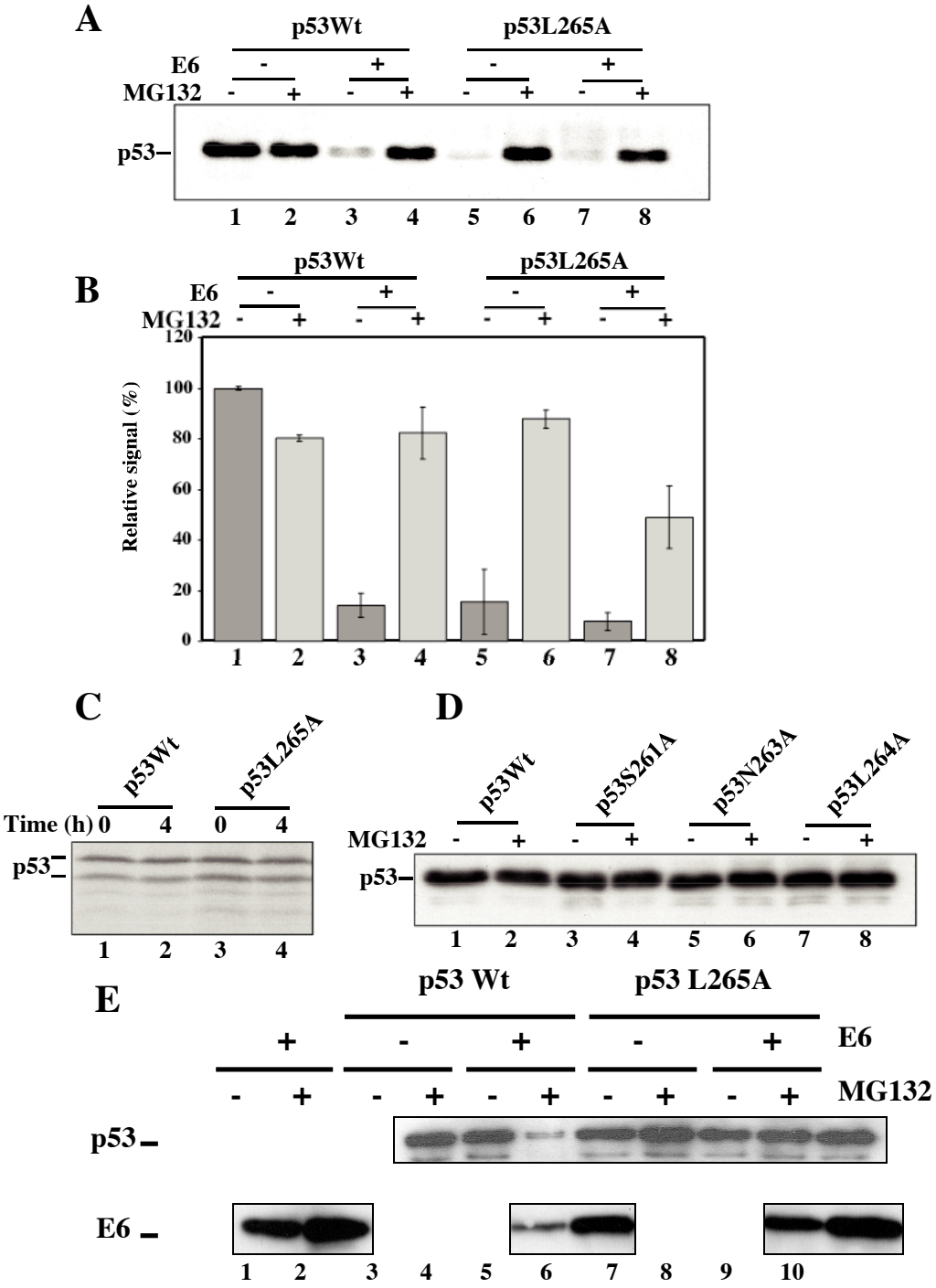

Figure 7.

The p53 mutant L265A is rapidly degraded in Saos-2 cells.

(A) Saos-2 cells were co-transfected by vectors for transient expression of 16E6 and p53 proteins. Where indicated, 16h prior to harvesting the cells, the culture medium was supplemented with the 26S proteasome inhibitor MG132 at a final concentration of 5 µM. 48h post-transfection cells were lysed. Volumes of lysate corresponding to a standardised quantity of total protein were then incubated with the Pab1801 monoclonal antibody conjugated to agarose beads. The immunoprecipitated material was then separated by 10% SDS-PAGE and subjected to Western-blotting using polyclonal antibodies raised against p53. (B) represents the mean ± standard deviation for three independent experiments.(C) ^{35}S-labelled p53 proteins produced in rabbit reticulocyte lysates before and after 4h incubation at 28°C and treated as described in the previous figure. (D) Immunoprecipitation of p53 proteins transiently expressed in Saos-2 in the absence of 16 and treated as in (A). (E) H1299 cells were treated as for Saos-2 cells with the exception that crude cellular extracts were used.

p53 L265A mutant is destabilised in Saos-2 cells but stable in rabbit reticulocyte lysates and H1299 cells

In order to determine the importance of the 261-265 region of p53 *in vivo*, we performed experiments using the p53-null cell line Saos-2, a cell line derived from an osteosarcoma. Cells were transfected with the indicated expression vectors and subjected to immunoprecipitation using agarose-conjugated Pab1801 anti-p53 antibody whereas for H1299 cells, crude cell extracts were used. In Saos-2 cells, Wt p53 protein was refractory to degradation in the absence of 16E6 and rapidly degraded in its presence (Fig. 7 A and B, lanes 1 and 3) whereas mutant p53 L265A was readily degraded both in the absence and presence of 16E6 (Fig. 7 A and B, lanes 5 and 7). Treatment of the cells with the 26S proteasome inhibitor MG132 had little effect on the Wt p53 protein level in the absence of 16E6 and restituted protein levels of Wt p53 in the presence of 16E6 (Fig. 7 A and B, lanes 2 and 4) while L265A p53 protein levels were augmented both in the presence and absence of 16E6 (Fig. 7 A and B, lanes 4, 6 and 7). Treatment with MG132 arrests protein degradation and thus proteins designated for proteasome-mediated degradation accumulate in the cells. These data indicate that, in the Saos-2 cells, L265A p53 is rapidly and contitutively degraded in a 26S proteasome dependant manner, independently of 16E6. In rabbit reticulocyte lysates described previously, Wt and L265A p53 proteins however, demonstrate comparable degradability in the absence of 16E6 (Fig. 7 C). In Saos-2 cells all the other p53 mutants (p53-S261A, -N263A, and -L264A) are also stable (Fig. 7 D). Transfection experiments of Saos-2 cells is of low efficiency and in order to generate quantifiable results in western blotting, immunoprecipitation of p53 from large quantities of cellular material was required. Additionally, immunoprecipitation of E6 from these cellular lysates was found to be unproductive as E6 remained undetectable. For H1299 cells, on the other hand, these were efficiently transfected, allowing us to use crude cellular extracts and to probe for 16E6 protein by western blotting (Fig. 7 E). As it is expected, Wt p53 protein is not readily degraded in the absence of 16E6 and rapidly degraded in its presence in a 26S proteasome-dependent fashion (Fig. 7 E, lanes 3 to 6). Interestingly, the mutant p53 L265A is stable both in the absence and in the presence of 16E6 and that MG132 has little to no effect on protein levels (Fig. 7 E, lanes 7 to 10). This suggests that the mutant L265A is less sensitive than Wt p53 to 16E6-mediated degradation in H1299 cells. 16E6 protein levels seem largely dependent on MG132 treatment (Fig. 7 E, lanes 1 and 2) but when associated with Wt p53, 16E6 level is lower (Fig.

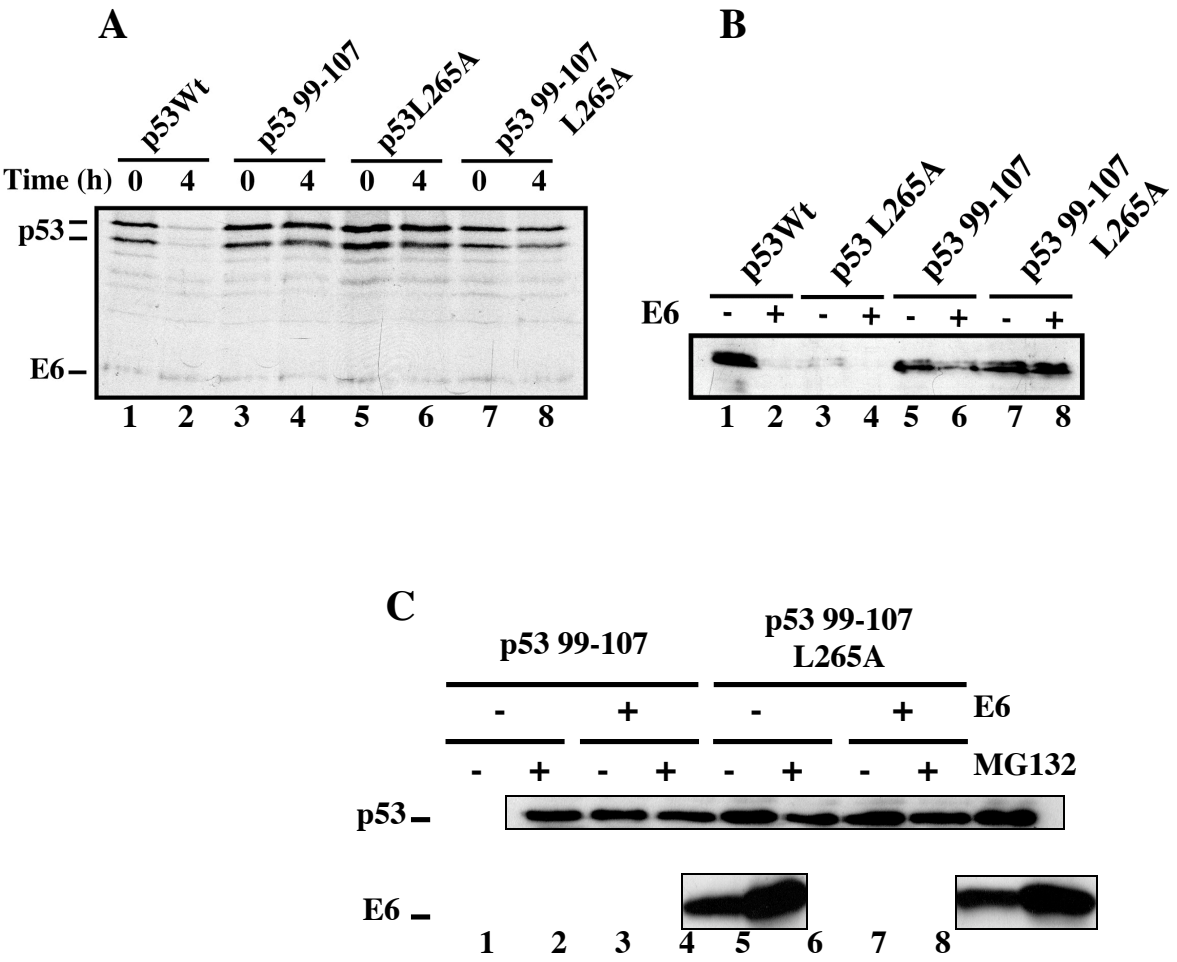

Figure 8.
The region 99-107 of p53 is critical for degradation.
(A) Radiolabelled p53 and 16E6 proteins produced in rabbit reticulocyte lysates were incubated together at 28°C and aliquots removed at the indicated times before separation by 12% SDS-PAGE and exposition to photographic film. (B) Saos-2 cells were co-transfected by vectors for transient expression of 16E6 and p53 proteins. Where indicated, 16h prior to harvesting the cells, the culture medium was supplemented with the 26S proteasome inhibitor MG132 at a final concentration of 5 µM. 48h post-transfection cells were lysed. Volumes of lysate corresponding to a standardised quantity of total protein were then incubated with the Pab1801 monoclonal antibody conjugated to agarose beads. The immunoprecipitated material was then separated by 10% SDS-PAGE and subjected to Western-blotting using polyclonal antibodies raised against p53. (C) H1299 cells were co-transfected by vectors for transient expression of 16E6 and p53 proteins. Where indicated, 16h prior to harvesting the cells, the culture medium was supplemented with the 26S proteasome inhibitor MG132 at a final concentration of 5 µM. 48h post-transfection, cells were lysed. Volumes of lysate corresponding to a standardised quantity of total protein were then separated by 10% SDS-PAGE and subjected to Western-blotting using polyclonal antibodies raised against p53.

7 E, lanes 5 and 6) and conversely, when associated with p53-L265A, the 16E6 level is higher (Fig. 7 E, lanes 9 and 10).

The region 261-265 interacts with the hydrophilic N-terminal arm (99-107) of the p53 core domain which is critical for p53 degradation

Using 3D representations of p53 core domain which encompass the DNA-binding domain, we noticed that the region spanning the amino acids 261 to 265 forms a loop that is in contact with a hydrophilic arm which marks the start of the core domain and spans the amino acids 95 to 108 (Fig. 1) and interaction takes place between the leucines 264 and 265 and the tyrosines 103 and 107. Interestingly, this region has previously been described as being required both for E6-dependant and E6-independant proteasome-mediated degradation of p53 (Gu et al., 2001). These structural considerations led us to mutate p53 in the N-terminal arm of the core domain by changing the amino acids ^{99}SQKTYQGSY^{107} to ^{99}GAGAGAGAG^{107}. This mutation was also combined with the L265A mutation to create the p53 proteins p53-99-107 and p53-99-107 L265A which were tested for their capacity to be degraded by 16E6 both *in vitro* and *in vivo* (Fig. 8). Both Wt p53 and L265A mutant behaved as previously observed (Fig. 8 A, lanes 1, 2, 5 and 6; B, lanes 1 to 4). p53-99-107 and p53-99-107 L265A, on the other hand, were resistant to degradation mediated by 16E6 in reticulocyte lysates (Fig. 8 A, lanes 3, 4, 7 and 8), Saos-2 cells (Fig. 8 B, lanes 5 to 8), and in H1299 cells (Fig. 8 C). The introduction of the mutation 99-107 to the p53 L265A mutant stabilised the protein when expressed in Saos-2 cells. Therefore, the 99-107 mutation seems to be a dominant stabilising feature for the degradation of p53 (Fig. 8 B, lanes 7 and 8) indicating that the 99-107 region is required for both the 16E6 and non-16E6 mediated degradation of p53. Additionally, we observed that the level of 16E6 was again closely related to the stability of the p53 protein. This correlation between E6 stability and p53 stability is further highlighted by the p53-99-107 and p53-99-107 L265A mutants (Fig. 8 C, lanes 3, 4, 7 and 8).

9.6 Discussion

The E6 proteins of high-risk HPV types are known to bind cellular proteins through a linear, α-helical, charged leucine motif. We employed phage-peptide display to investigate E6-binding ligands. The peptides isolated provide information of conformational and non-conformational binding interfaces. The 165 peptide sequences resulting from the phage-display experiments on 16E6 (6S/6C) are currently the subject of investigation in our laboratory and, although the exact significance of all the selected peptides has yet to be determined, two groups attract immediate comment. The Group 1, which was generated from phage display experiments using plastic-immobilised 16E6 (6S/6C), consists of highly hydrophobic peptides for which a consensus motif could be allocated, suggesting specificity. However, when the same library was used on 16E6 (6S/6C) protein in suspension attached to sepharose beads this family was not isolated, suggesting that these are probably plastic-binders.

Group 7 is of particular interest as it is composed of peptides that have a consensus motif consisting of SXNLLG. This is strikingly similar to the LXELLG consensus motif obtained by Elston *et al.* but shares greater homology with the p53 primary sequence ^{261}SGNLLG^{266} (Elston et al., 1998). Furthermore, "charged leucine" motifs adopt an α-helix conformation in full-length proteins (Savkur and Burris, 2004). On the other hand, in the p53 protein core domain, this region folds mainly as a loop, stabilised into a conformation by adjoining amino acids. These considerations led us to study the ^{261}SGNLLG^{266} region of p53 in 16E6 binding and 16E6-mediated degradation.

p53 L265 is required for 16E6 binding and degradation

It was found that E6 bound both a peptide spanning the region 256 to 267 of p53 (p53-peptide) and the "NLLG"-peptide isolated by phage display. This strongly suggests that this region is implicated in 16E6 binding. Additionally it was of great interest that we observed resistance to E6-mediated degradation for the p53 L265A mutant both *in vitro* in rabbit reticulocyte extracts and *in vivo* in H1299 cells. The importance of L265 for 16E6 binding and 16E6-mediated degradation of p53 may be explained by its interaction with Y107. Indeed, it has been reported that the region 92 to 112 of p53 confers susceptibility to E6 and MDM2-mediated degradation to p73 that is normally bound but not degraded by these proteins (Gu et al., 2001). Comparing the homologous regions 92 to 112 of p53 with 106 to 130 of p73, it is of note that the Y107 found in p53 is not conserved. The pivotal role for L265 in 16E6 binding to the region 261-265 of full-length p53 protein and its subsequent degradation may be due to a local conformation determined by the L265-Y107 interaction. On the other hand, we observed that amino acids S261 to L264 that may be less important for this conformation as their mutation did not affect the degradation of p53. This structural requirement for E6 binding may be precluded by the mutation L265A as 16E6 is able to bind the free peptide representing the peptide composed of the amino acids 256 to 267 of p53 (p53-peptide). The mutation L265A partially abrogated 16E6-binding suggesting that E6 was still able to bind the C-terminal domain of p53.

p53 L265A is rapidly degraded in Saos-2 cells

The behaviour of the p53 mutant L265A Saos-2 cells is markedly distinct from that observed in either H1299 cells or reticulocyte lysates. This points to a fundamental difference in the three systems used to investigate this mutant as it remained stable when transiently expressed in H1299 cells and when produced in reticulocyte lysates. p53 is regulated in normal cells by E3-Ub ligases, the most studied of these is MDM2 (Mouse Double-Minute 2) yet others have been isolated more recently including Pirh2 (Leng et al., 2003), COP1 (Constitutively photomorphogenic 1) (Dornan et al., 2004b) and Topors (Rajendra et al., 2004). Over expression of MDM2 (Bode and Dong, 2004), Pirh2 (Duan et al., 2004) and COP1 (Dornan et al., 2004a) has been associated with increased degradation of p53 in cancers. Our results suggest that this is not the case for Saos-2 cells as when these were transfected with Wt p53, the protein remained stable. This was also observed for reticulocyte lysates and H1299 cells and is substantiated by independent studies in which co-expression of MDM2 (Zeng et al., 1999), Pirh2 (Leng et al., 2003) or Topors (Rajendra et al., 2004) is required to induce the degradation of transiently expressed p53 in either Saos-2 or H1299 cells. Therefore, it is unlikely that the increased susceptibility of L265A p53 mutant to degradation in Saos-2 cells is directly linked to the amplification or increased expression of one of these E3-Ub ligases. Furthermore, the results presented here suggest that degradation of L265A in Saos-2 cells is mediated by a cellular factor present in these cells that is not functionally active and/or present to sufficient quantities in either rabbit reticulocyte lysates or H1299 cells. This is particularly interesting and elucidation of the factor responsible for this observation may greatly contribute to the understanding of p53 degradation.

One candidate which could be the cellular factor responsible for the increased degradation of L265A mutant p53 in Saos-2 is the CSN complex. Indeed, it has been demonstrated that a sub unit of COP9 Signalosome (CSN), CSN 5, binds to p53 in the region spanning the amino acids amino acids 1 to 154 (Bech-Otschir et al., 2001). Furthermore, both the CSN 5 and CSN 2 subunits have been implicated in the degradation of p53 (Bech-Otschir et al., 2001; Chang and Schwechheimer, 2004; Lykke-Andersen et al., 2003). Interestingly, Saos-2 cells were derived from an osteosarcoma and in many of these carcinomas, another CSN sub unit, CSN 3, has been found to be over expressed, allowing the possibility that the whole complex is also over expressed (Henriksen et al., 2003). The CSN complex is

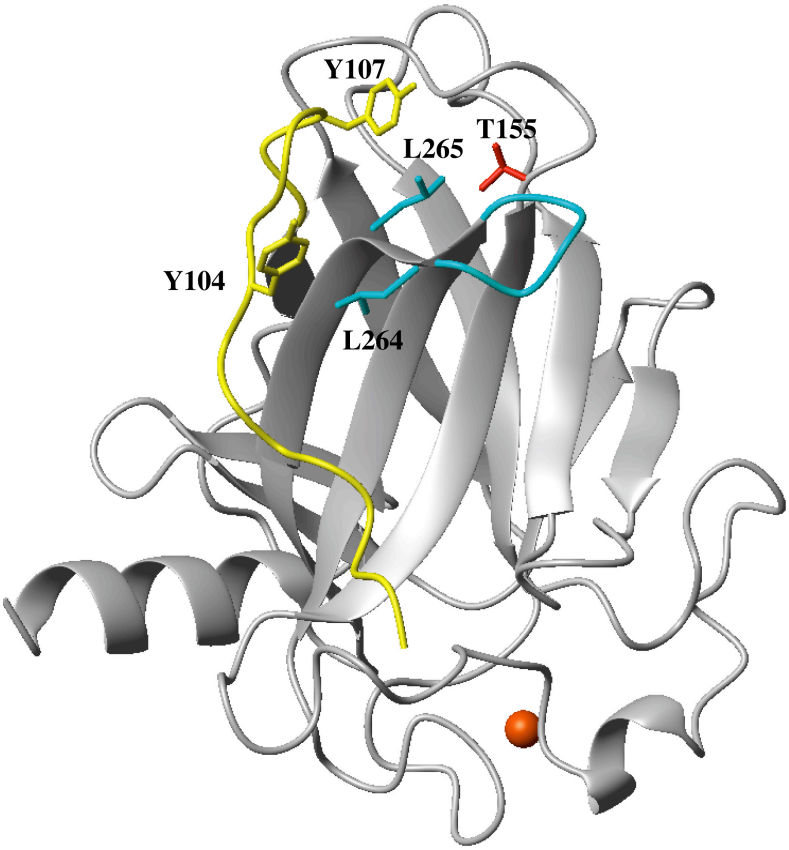

Figure 9.
Representation of the p53 core domain tertiary structure.
This is the same representation as described in Fig. 1 rotated to show more clearly the contact made by Y107, T155 and L265.

composed of 11 subunits that demonstrate sequence homology to the subunits composing the 19S lid of the 26S proteasome (Schwechheimer, 2004). It is suggested that the CSN complex may be capable of replacing the 19S lid of the 26S proteasome. The 19S lid recognises polyubiquitinated substrates and de-ubiquitinates these before the 19S base unfolds and feeds the protein to be degraded into the 20S core. Thus, the CSN complex may play a role in regulating or mediating degradation of proteins by the 26S proteasome. This hypothesis is further supported by the observations that overexpressed CSN 2 was found to be in the 19S complex and that the CSN 5 is capable of de-ubiquitination, required for proteolysis in the 20S proteasome (Chang and Schwechheimer, 2004).

It is proposed that, in normal cells, CSN may play a regulatory role for p53 degradation by mediating its phosphorylation. Indeed, CSN-associated kinases are reported to phosphorylate p53 in the region 94 to 103 and the amino acids S149, T150 and T155 and the p53 mutant T155V was found to be resistant to MDM2 and E6-mediated p53 degradation (Bech-Otschir et al., 2001). This suggests that phosphorylation of T155 therefore is a prerequisite for MDM2 and E6-mediated degradation but is not sufficient to induce degradation independently of ubiquitin ligase function. Observation of the structure of the p53 core domain shows that T155, Y107 and L265 are in close proximity (Fig. 9) and it could be envisaged that phosphorylation of T155 causes a conformational change in the core domain.

We propose a mechanism in which the CSN binds to p53 in the region 1-154, CSN-associated kinases then phosphorylate a residue, possibly T155, causing a permissive conformational change required for MDM2 or E6 mediated degradation. Mutation of L265 in the p53 protein not only precludes 16E6 binding but causes release of the hydrophilic arm 99-107 which can then interact more readily with the CSN complex associated with or replacing the 19S lid and thus induce degradation in an ubiquitin-independent manner. This is in agreement with the results presented here as mutation of the hydrophilic arm abrogated degradation of p53. Which is further supported by the report of a structural element required for degradation spanning the amino acids 101 to 131 of p53 (Li and Coffino, 1996b).

An interesting aside revealed in the work presented here is that the degradation of 16E6 is directly related to p53 degradation. When 16E6 is transiently expressed in cells it is rapidly degraded by the 26S proteasome and when co-expressed with wild-type p53 this degradation is increased. Conversely, when 16E6 is co-expressed with the L265A or 99-107

p53, degradation of 16E6 is abrogated. Degradation of high-risk E6 by the ubiquitin-26S proteasome system has previously been described and is in accordance with the results presented here (Kehmeier et al., 2002; Stewart et al., 2004). However, this is the first description of E6 degradation associated with E6-mediated degradation of p53. Whether this p53-dependant degradation of E6 is a result of increased ubiquitination of E6 or directly due to the degradation of p53 remains unanswered. An interesting prospective indicated by these results is that it is a p53/E6 complex that is degraded. However, results obtained using the rabbit reticulocyte lysates do not support this hypothesis, as E6 remains stable even when p53 is efficiently degraded. Another possibility is that the E6 protein level is due to the increased p53 protein level, suggesting that the p53 protein affects expression of E6 when transiently co-expressed. This will be an interesting point to develop in further studies using expression vectors comprising different promoters than those used in the study described here.

GENERAL CONCLUSION

The work presented here tackles two different aspects of cervical cancer one molecular and the other cellular. Indeed, the targeted delivery of therapeutical agents and the molecular mechanisms leading to transformation of HPV-infected cells are explored. In the first study, the biopanning procedure methodology employed was optimised for the selection of phage-peptide clones that bind and rapidly internalise into HPV16-transformed SiHa cells. This is contrasted by the second study where phage-peptides were affinity selected for binding to the 16E6 protein. However, although the selection criteria differ, the two methodologies present the same fundamental principle of biopanning in which successive rounds of selection enrich the isolated population with positive clones.

The tumour targeting of therapeutical strategies reduces side effects and increases efficiency. Here we have isolated peptides which demonstrate specificity for the cervical cancer cell lines transformed by high-risk HPVs (article A). These results are encouraging yet further investigation in animal systems is required for the evaluation of their efficiency. Indeed, the use of these peptides will be restrained to local application by injection in the tumour as opposed to injection into the general blood stream. This is due to the tumour vasculature being unlikely to present the same modifications as the tumourous epithelial cells targeted by these peptides. Using these peptides for the targeting of therapeutical molecules such as proapoptotic peptides or vehicles for the selective delivery of such molecules for example adenoviruses are currently the subject of investigation in our laboratory. Targeted material is internalised into cells and follows the endosomal-lysosomal system that results in degradation. Both adenoviruses and the proapoptotic peptides investigated in our laboratory have an inherent capacity to escape the endosomal system and degradation in the lysosome. This cellular fate must is addressed by the use of the diphtheria toxin. This protein is composed of three functional domains one of which, domain T, is responsible for translocation of the toxic A-domain across the endosomal membrane. Using a mutant form less toxic of the diphtheria toxin, we succeeded in replacing the third domain responsible for receptor-binding with one peptide for cervical cancer cell targeting. Yet, the results obtained with this construct did not demonstrate efficient translocation of the A-domain from endosome to cytosol. The results obtained may be due to an inherent incapacity of the diphtheria toxin to promote translocation of the A-domain. Alternatively, this may be due to the construction used in this study which may limit the efficiency of translocation. It is clear that the efficiency of translocation must be investigated before the translocation domain of the diphtheria toxin may be used for the cytosolic delivery of therapeutic molecules. If this

capacity of translocation is indeed limited, the translocation of the A-domain by the wild-type diphtheria toxin is sufficient to produce a toxic effect. Therefore, the targeting peptides described in article A may be used to redirect simply the toxicity of the wt diphtheria toxin as has been extensively described (Frankel et al., 2002). The diphtheria toxin may not provide an efficient mechanism for the delivery of structured polypeptides as a molten globule formation is required for movement through the T-domain yet small peptides may be easily translocated. Therefore, a more efficient strategy involving the capsid proteins of the adenovirus or adeno-associated virus which have endosomo-lytic activity may be investigated. Such strategies would not restrict the delivered molecule to simple peptides yet would circumvent the problems associated with the gene therapy strategies more familiar with viral vectors.

The transformation of infected cells by the high-risk HPVs is mediated by the two oncoproteins E6 and E7. Investigation of the molecular interactions of E6 has been one of the subjects of intense research in our laboratory that is of paramount importance in the understanding of the transformation process. Here, we isolated a set of peptides that have resulted in the isolation of a region within the p53 core domain important for its degradation mediated by high-risk E6. A second region, connected in the 3D representation of the core domain structure and essential for the degradation of p53 both dependent and independent of E6 was also identified. The finding that the mutation L265A in the p53 sequence results in a molecule resistant to degradation by 16E6 yet which promotes the rapid degradation of p53 in the cell line Saos-2 may be a fundamental finding for the understanding of the degradation of p53. Indeed, it will be of great interest to study the effect of this mutation on the degradation of p53 mediated by MDM2 and the other ubiquitin ligases more recently identified. The proposition made in the manuscript B that phosphorylation by the CSN-associated kinases mediates a conformational change may be an interesting lead for the isolation of the cellular factor(s) involved in the rapid degradation of this mutant in Saos-2. It will also be of great interest to define the transcription factor activity of this p53 mutant. Although the mutation L265A is distal to the DNA binding surface of the p53 core domain, the proposed conformational change induced by the mutation may alter this activity. However, if the functional activity of p53 is not affected and the mutant protein is resistant to other high-risk E6 oncoproteins then it could be envisaged that it might be capable of replacing wild-type p53 in HPV-transformed cells.

The other results generated by the biopanning experiments on 16E6 protein have yet to be fully exploited. Linear stretches of amino acids homologous to the other peptides isolated were not found in other known cellular protein partners of E6. However, these may provide information of conformational, non-linear binding surfaces. It would be interesting to test the isolated peptide sequences for the inhibition of binding between 16E6 and its known cellular partners that would provide information regarding the interacting surfaces. For those partners known to interact through charged leucine motifs or PDZ domains, the interacting surfaces may be more extensive than first thought. Furthermore, these peptides may be interesting leads for the destabilisation of the 16E6 binding to its cellular partners and therefore, the inhibition of its activity as has been previously described (Butz et al., 2000). If such an activity were detected, it would be interesting to develop these peptides along with the intracellular delivery vehicle based on the diphtheria toxin.

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APPENDIX

TITRE: Cancer du col de l'utérus: Adressage tumoral et définition de ligands peptidiques de l'oncoprotéine E6 de HPV16.

RESUME: Ces 20 dernières années, l'importance des virus du papillome humain (HPV) dit de "haut risque" dans le développement du cancer a été bien documentée, en particulier dans le cancer du col de l'utérus. Les deux oncoprotéines virales E6 et E7 participent à de nombreux processus cellulaires conduissant à l'immortalisation des cellules. En particulier, le processus de degradation de p53 médié par E6/E6-AP n'est pas connu dans le détail. Le décryptage du mécanisme moléculaire de l'interaction de E6 de HPV de haut risque avec ses partenaires cellulaires fournira à terme des donnés indispensables dans la compréhension du processus d'immortalisation et le développent d'inhibiteurs spécifiques et efficaces. L'élaboration de stratégies de ciblages aidera aussi bien le diagnostic que le traitement efficace des tumeurs. L'objectif de mon travail de doctorat est articulé autour de deux axes. Le premier a eu trait a la sélection par phage display de peptides ciblant spécifiquement des cellules issues de tumeurs du col de l'utérus. Ces peptides ont été ensuite utilisés pour re-diriger la toxine diphtérique spécifiquement au niveau de cellules SiHa, lignée cellulaire issue d'un carcinome cervical humain. Le deuxième axe a concerné la définition par phage display de plusieurs familles de ligands peptidiques de E6 de HPV 16. Une des familles de peptides présente une homologie significative avec une région de p53 . L'étude approfondie in vitro et sur cellules en culture de mutants de p53 dans cette région a révélé son importance dans la dégradation de p53 par E6.

DISIPLINE: Biologie Moléculaire

MOTS CLEFS: oncoprotéine E6, HPV, cancer du col de l'utérus, phage display, ciblage cellulaire, p53, dégradation.

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