UV response of mammalian p53 tumor suppressor gene

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Academic Dissertation

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To Mum,
who never ceased to love life

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ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Haapajärvi, T., Kivinen, L., Pitkänen, K., and Laiho, M. Cell cycle dependent effects of UV-radiation on p53 expression and retinoblastoma protein phosphorylation. *Oncogene* 11: 151-159, 1995.

II Haapajärvi, T., Pitkänen, K., Tsubari, M., and Laiho, M. p53 transactivation and protein accumulation are independently regulated by UV light in different phases of the cell cycle. *Mol. Cell. Biol.* 17: 3074-3080, 1997.

III Haapajärvi, T., Kivinen, L., Heiskanen, A., des Bordes, C., Datto, M., Wang, X-F., and Laiho, M. UV radiation is a transcriptional inducer of $p21^{Cip1/Waf1}$ cyclin-kinase inhibitor in a p53 independent manner. *Exp. Cell. Res.* 248: 272-279, 1999.

IV Haapajärvi, T., Pitkänen, K., and Laiho, M. Human melanoma cell line UV responses show independency of p53 function. *Cell Growth & Diff.* 10: 163-171, 1999.

ABBREVIATIONS

AP	apurinic/apyrimidic
ARF	alternative reading frame
ATM	ataxia telangiectasia mutated
ATR	ATM related
bp	base pair
5-BrdUrd	5-bromo-2´-deoxyuridine
CAT	chloramphenicol acetyltransferase
Cdk	cyclin dependent kinase
CIP1	Cdk inhibiting protein 1
CK	casein kinase
CKI	cyclin kinase inhibitor
CS	Cockayne´s syndrome
DNA-PK	DNA-dependent protein kinase
EMSA	electrophoretic mobility shift assay
ERCC	excision repair cross complementing
FCS	fetal calf serum
GADD	growth arrest and DNA damage inducible
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPV	human papilloma virus
hsp	heat shock protein
HU	hydroxyurea
IGF-BP	insulin-like growth factor binding protein
IL	interleukin
INK4	inhibitor of Cdk4/6
JNK	jun amino-terminal kinase
LFS	Li-Fraumeni syndrome
MAP	mitogen activated protein
MDA	melanoma differentiation associated protein
mdm2	murine double minute gene 2
MEF	murine embryo fibroblast
MeOH	methanol
mRNA	messenger ribonucleic acid
NBCS	newborn calf serum
NER	nucleotide excision repair
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PI3-K	phosphatidylinositol 3-kinase
pol	polymerase
RB	retinoblastoma
RT-PCR	reverse transcription-polymerase chain reaction
SDI1	senescent cell-derived inhibitor 1
SCID	severe immunodeficiency
SV	simian virus
TAF	TBP-associated factor
TBP	TATA box-binding protein
TBS	Tris-buffered saline
TGF	transforming growth factor
TTD	trichothiodystrophy
UV light	ultraviolet light
VEGF	vascular endothelial growth factor
WAF1	wild-type p53 activated factor 1
XP	xeroderma pigmentosum

SUMMARY

To prevent accumulation of genetic lesions, cells subjected to genotoxic stress have two equally important ways to respond. Most commonly, DNA damage elicits a replicative arrest at various cell cycle checkpoints in order to give the cell time to accomplish DNA repair. Alternatively, in the case of intolerable or unrepairable DNA lesions upon excessive insult, apoptosis remains the only possibility to remove genetically damaged material, which may otherwise predispose the cell to genomic instability and cancer.

The ability of p53 tumor suppressor to inhibit malignant transformation and cancer formation has been addressed as far as its capability to control cell cycle progression and induction of apoptosis. A role of p53 as a mediator of growth arrest and apoptosis has been suggested, since it is well established that after γ-irradiation, and many other DNA damaging stimuli, p53 is needed for normal cellular response. At the beginning of our study, little was known about the activation pattern and requirement of p53 after UV radiation, which causes DNA damage very different from those generated by ionizing radiation.

To study the action of p53 in UV-damaged cells, a fruitful approach turned out to be UV treatment of mouse fibroblasts synchronized to a particular cell cycle phase. This method allowed us to explore in detail the UV-induced cell cycle responses and cell cycle phase specific activation of p53 as well as other regulators of proliferation. Contrary to expectations, p53 accumulation failed to occur in UV-treated, G1-phase arrested cells, but accumulation was observed when the cells recovered from the G1 arrest and entered the S phase or, alternatively, when the cells were irradiated and subsequently arrested in S. Rather than being dependent on the cell cycle phase as such, p53 accumulation in G1/S and S phases appeared to be dependent on replication of damaged DNA. Furthermore, pRB hypophosphorylation, which was shown to occur independently of functional p53, correlated with the kinetics of both general UV-triggered growth arrest and specific G1-phase arrest. Further studies indicated, however, that despite absent p53 accumulation p53 had a transactivation function in all cell cycle phases. Thus, in contrast to replication-dependent accumulation of p53 its UVC-mediated transactivation seemed independent of cell cycle phase and protein stabilization.

In contrast to the majority of cancers, p53 mutations are seldom detected in human melanomas. Of seven melanoma cell lines studied, three carried a mutation, and cell lines harboring normal p53 expressed high levels of wild-type p53, another special feature of p53 in melanomas. Although all p53 target genes (p21, GADD45, and mdm2) were induced upon UV in cells expressing wild-type p53 – and most of them also in cell

lines with mutant p53 cell lines – their induction was dissociated from p53 function. Interestingly, GADD45 induction, but not p53, correlated with growth arrest and induction of apoptosis. In addition to abnormal stabilization of wild-type p53, many aspects of the p53 pathway showed abnormalities, and UV responses did not significantly differ among cells carrying mutant or normal p53 suggesting functional inactivation of wild-type p53 in melanoma cell lines.

Since previous studies were highly suggestive of p53-independent regulation of p21 cyclin-kinase inhibitor, UVC responses and p21 activation were examined in p53-/- mouse fibroblasts. UVC radiation induced rapidly and efficiently both protein and mRNA levels of p21 also in the absence of p53. In contrast to p53+/+ MEFs, however, high UVC doses abrogated p21 protein induction in p53-deficient cells, and secondly, p53-/- cells were more prone to apoptosis, suggesting a requirement of p53 function in these responses. p21 promoter assays confirmed transcriptional activation of p21, and UV-inducibility was mapped to two regions in the p21 promoter, both lacking p53-binding sites.

These studies have particularly shed light on the cell cycle phase-specific regulation of p53 accumulation and transactivation in UVC-damaged cells. In addition, by using several different approaches, p21 induction upon UV treatment was confirmed to occur at the transcriptional level also independently of p53. Finally, despite a wild-type coding sequence of p53 several p53 regulatory steps were proved to be disturbed in melanoma cell lines suggesting that defective function of p53 may, after all, play a role also in this cancer type.

INTRODUCTION

Cancer is a disease characterized by a loss of normal control of cell growth. Uncontrolled growth of malignant cells results from their disability to respond to extracellular cues leading either to growth arrest, differentiation, or apoptosis. After transformation the malignant cell proliferates independently ignoring the signals from the environment. Several different alterations in multiple pathways can cause genomic instability, and according to a multistep theory one harmful genetic change predisposes the cell to another, parallel mutation. Even if loss of one cell growth regulator could be succesfully compensated, two or more changes may permanently disturb the growth control and give rise to clinical cancer. Activation of proto-oncogenes, inactivation of tumor suppressors and defective DNA repair processes are all mechanisms that disrupt the control of cell division and potentially lead to accelerated, inappropriate cell proliferation.

Compared to oncogenes, the study of tumor suppressors has proved to be quite difficult. While one mutated allele of an oncogene is usually sufficient for altering the phenotype, the recessive nature of most tumor suppressors requires the inactivation of both alleles. Moreover, by the definition, growth suppressors inhibit growth making their identification even more difficult. At present a dozen tumor suppressor genes have been identified; the retinoblastoma gene product (pRB), p53, and more recently, some cyclin kinase inhibitors, are most extensively studied.

pRB, which is at the interphase of the cell cycle machinery and transcriptional control mechanisms, represents a classic recessive tumor suppressor, while mutation in one allele of p53 gene may suffice for cancer formation. p53 mutations, often in conjunction with other cellular changes, are detected in about 60% of all human cancers. As a transcription factor p53 has the potential to affect diverse cellular processes via target gene activation, and its role in DNA-damage induced growth arrest, induction of apoptosis, and DNA repair processes is well established. Because p53 is involved in cell cycle checkpoint control, its activation by different DNA damaging and growth arresting conditions is of special interest.

p53 protein accumulates in response to diverse genotoxic insults. p53-mediated apoptosis and growth arrest due to cytotoxic drugs and ionizing radiation have represented the main streams for exploring p53 function. At the beginning of our studies the need and requirement of p53 function for UV-radiation caused growth arrest and apoptosis were undefined, but it was generally assumed that UV, although causing DNA lesions totally different from those upon γ-irradiation, elicites a similar response to damage. Similarly, despite the evident participation of p53 in the regulation of cell cycle progression, little was known about the cell cycle phase-dependent activation of

p53. In earlier studies p53 target genes appeared to be under strict control of intact p53, but later these proteins, all involved in cell cycle control, apoptosis, or DNA repair, were found to be regulated by multiple pathways.

Rather than studying the p53 conformation and downstream events the focus in p53 research has now been turned to exploring specific regulation of p53, the events that earlier practically nothing was known about. Due to its apparent clinical importance, p53 has been one of the most vigorously studied molecules in the field of cancer molecular biology, and even now, after twenty years of research, the interest still shows no signs of subsiding.

REVIEW OF THE LITERATURE

CELL CYCLE CONTROL AND CANCER

Unlimited growth, the most characteristic feature of tumors, represents the sum of unfavourable events. First, a genetic change, due to intrinsic or extrinsic signals, has taken place and secondly, recognition of DNA damage or its repair process has failed. Once genomic instability has arisen, additional changes accumulate more easily. Later, selection favours malignant cells with increasingly aggressive growth properties and allows rapid expansion of the tumor. Although a cell has diverse cautionary measurements to prevent loss of growth control, some of them may fail. Most commonly, cancer results from tumorigenic abberrations of cell cycle regulators, in particular, those governing the G1 progression and G1/S-transition.

Cell cycle regulators

Cells, tumor or normal cells, follow the steps of cell cycle consisting of G1, the actual growth phase during which they prepare to synthesize DNA; S, DNA replication; G2, a second shorter growth phase; and M, mitosis. Cells that have transiently or permanently exited the cell cycle, for example because of serum starvation, terminal differentiation, or senescence, remain in the G0 phase. Cells are constantly a target for mitogenic and antimitogenic signals that affect them only during the G1 phase. Growth factors are required for progression through G1 to a specific point called the restriction point (R-point), after which the cell is committed to complete the division cycle even in the absence of growth factors thereafter (Pardee, 1989) (Fig. 1).

The role of cyclins and cyclin-dependent kinases (Cdks) in the precise control of cell cycle is well established. Mitogenic signals cause the assembly of different kinase holoenzymes, that are composed of a cyclin regulatory subunit and a Cdk catalytic subunit. These complexes are formed and activated at specific phases of the cell cycle, and their coordinated function is required for progression through the S and M phases. Contrary to cell cycle phase-specific expression of cyclins, Cdks are expressed constitutively, but are not active until complexing with suitable Cdk. Additionally, the kinase activity of certain Cdks can be regulated by phosphorylation of critical residues, or kinase activity inhibited by various Cdk-inhibitors (CKI), some of which have been shown to possess tumor suppressive properties. p21, p27 and p57 are universal Cdk-inhibitors with broad specificity for Cdks, whereas the INK4 (inhibitor of Cdk4/6)

family members p15, p16, p18, and p19 selectively inhibit only the kinase activity of Cdk4 and Cdk6 in the G1 phase (Kamb, 1995).

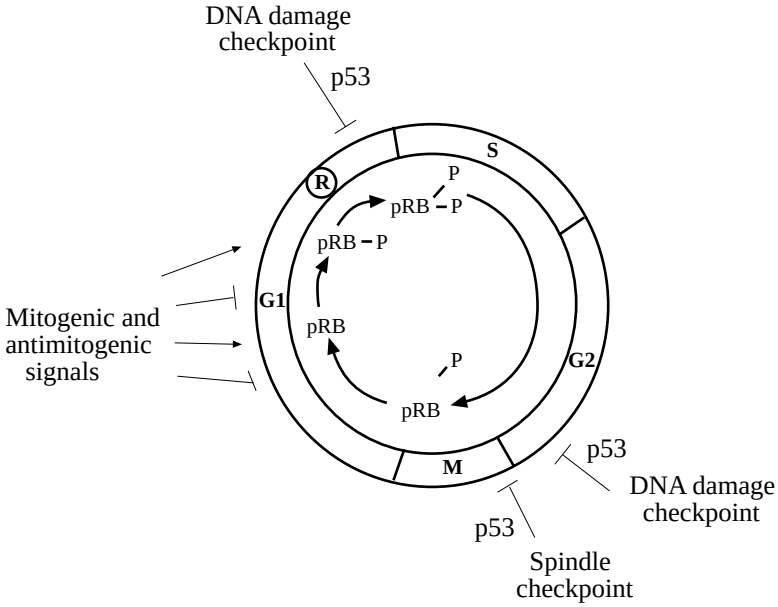

Fig. 1. Cell cycle phases. p53 functions at all checkpoints, while pRB activity varies in a cell cycle phase dependent manner. R= Restriction point, ⟶ = activation, —| = inhibition. Modified from Grana and Reddy, 1995.

The tumor suppressor gene p53 and the retinoblastoma susceptibility gene (RB) are both involved in the regulation of the cell cycle and are closely linked to cyclin-Cdk pathway. While retinoblastoma protein (pRB) is required in the control of normal progression of the cycle, p53 is called into action in the case of genetic insult. pRB is phosphorylated in a cell cycle dependent manner and is in the hypophosphorylated, active form in the early G1 and binds transcription factor E2F-1. Phosphorylation of pRB by cyclin D-Cdk4/6 in the late G1 results in the release of E2F-1, which activates genes required for the progression to the S phase (Fig. 1.). At present, pRB is the only known target of cyclin-Cdk activity in G1.

The antitumorigenic potential of p53 correlates with its ability to check the integrity of the genome and cease the progression of the cell cycle when DNA damage is encountered. Upon genetic insult, p53 transactivates, among other target genes, the expression of p21 Cdk-inhibitor and thus participates in the growth arrest of cells (Fig. 3). p53, often called the guardian of the genome, is the most often mutated gene in human cancers, stressing the importance of p53 and cell cycle control in tumorigenesis. Whatever the mechanism, increased activity of cyclins and Cdks as well as decreased function of CKIs, pRB, and p53, highly predispose cells to transformation.

Cellular response to DNA damage

Cells are constantly exposed to both extrinsic and intrinsic DNA damage signals. Extrinsic sources of damage include irradiation and chemical mutagens, while intrinsic damage is generated by the cell itself. Despite the high spontaneous chemical reactivity of DNA, only one error is made during replication of the $3x10^9$ bases of human genome. Proliferating cells have a great capacity to tolerate genomic errors, as cells can repair about 100 spontaneous alterations per hour (reviewed by Lehmann and Carr, 1994).

DNA damage types can be grouped into two main categories: damage modifying nitrogenous bases, and damage alterating the phosphodiester backbone. Chemical agents can be covalently attached to bases making intrastrand or interstrand cross-links or cause depurination or depyrimidination of DNA (reviewed by Friedberg, 1995). Ultraviolet (UV) radiation causes special kinds of changes: cyclobutane pyrimidine dimers and (6-4) photoproducts. These lesions are repaired by photoreactivation of DNA and by nucleotide excision repair (NER). Most DNA lesions produced by free oxygen radicals are corrected by base excision repair which is distinct from NER. In this repair system the damaged base is cleaved from its deoxyribose moiety and the apurinic or apyrimidic (AP) sites are excised followed by DNA repair synthesis and ligation. AP sites can also result from spontaneous depurination or depyrimidination of DNA. Mismatch repair, on the other hand, refers to correction of mispaired bases, which are frequently produced during replication and recombination. It can occur by several biochemical pathways including base excision (reviewed by Friedberg, 1995). Broken phosphodiester bonds, on the other hand, cause double-stranded breaks which are repaired by nonhomologous end-joining or by the addition of new telomeres (Wilkie et al., 1990).

Cells have a number of systems to interrupt cell cycle progression when damage to the genome is detected or when cells have failed to complete the preceding cell cycle phase. The DNA damage checkpoint can be regarded as a signaling system, where information flows from factors detecting DNA lesion to cell cycle targets. Signals activating the checkpoints can be produced from recognition of DNA damage itself, during repair, or during replication of damaged sites (Lehmann and Carr, 1994). DNA damage checkpoints act at the G1/S border, during S, and at the G2/M boundary. Although arrest points are scattered throughout the cell cycle, the same or similar proteins are often involved in all of them. In addition, many of these proteins function not only in checkpoint control but also in DNA repair, apoptosis, and transcriptional induction (Paulovich et al., 1997). p53, for example, has a role in each DNA damage checkpoint, in spindle checkpoint, and in apoptosis.

The desired outcome of DNA damage checkpoint control is either a successful repair of DNA after growth arrest, or, in the case of permanent damage, induction of apoptosis. In both these situations DNA errors are not mediated onwards, whereas replication of damaged DNA leads to accumulation of mutations or genomic instability. In addition to the intrinsic error rate, the checkpoint control system may fail because of adaptation or selective pressure. In adaptation, the cell cycle continues after an interval of arrest even if the damage remains unrepaired (Sandell et. al., 1993). In some pathological conditions, like cancer, selection favours cells with defective checkpoints, since genomic instability gives rise to multiple genetic changes needed both for the primary transformation and for increased invasiveness (reviewed by Paulovich, 1997).

UV RADIATION

UV radiation can be divided into three main classes according to its wavelength; UVA (320-400 nm), UVB (290-320 nm), and UVC (100-290 nm). Because the atmospheric ozone layer efficiently absorbs the shortest waves of UV radiation, solar light consists only of UVA and UVB. Even if human skin never meets UVC light, the same DNA lesions, although at a lower efficiency, are produced after longer wavelengths of UV. Besides typical UV-induced lesions, UVA/B also causes considerable oxidative damage. Being usually easily available and measurable, UV radiation, especially UVC, has been quite extensively used in studies of DNA damage and repair. When DNA damage studies are evaluated, the nature of genetic insult is of great importance, as the damage response differs between DNA damage types.

DNA lesions induced by UV radiation

UV radiation causes the formation of a specific type of DNA lesions, the "UV footprints". When DNA is exposed to UV, adjacent pyrimidines become covalently linked by the formation of four-membered ring structures, referred to as cyclobutane pyrimidines or pyrimidine dimers. These lesions distort the helical structure of DNA, and some isomeric forms, but not all, are able to obstruct DNA replication and transcription (Taylor et al., 1990). Formation of pyrimidine dimers is a reversible process, and with high UV doses an equilibrim between the formation and dissociation of dimers by photoreversal can be reached. The dimer formation seems not to take place randomly between pyrimidines, but thymine containing dimers are preferred over cytosine containing ones. With UVC of 254 nm the ratio of T-T to C-T to T-C to C-C was found to equal 68:13:16:3 (Tornaletti et al., 1993; Mitchell et al., 1992).

Another typical UV-induced DNA lesion is the formation of pyrimidine-pyrimidone (6-4) photoproducts, most often of the TC and CC types, which considerably distort the DNA helix (Taylor et al., 1988). Generally the incidence of photoproducts after UV radiation is several times lower than that of pyrimidine dimers, although at occasional sites in DNA (6-4) lesions may occur as frequently as dimers (Bourre et al., 1987) . However, the frequency of UV-induced nonsense mutations correlates better with the frequency of photoproducts than of pyrimidine dimers, suggesting that (6-4) lesions may have a stronger contribution to the mutagenicity of UV radiation (Mitchell et al., 1989).

Although UV and γ-radiation seem to cause quite similar cellular responses, growth arrest and apoptosis, the nature of DNA damage is quite different. The most striking lesions made by ionizing radiation are direct single and double strand breaks, which are almost never caused directly by UVC. However, DNA-protein cross-links and DNA strand breaks are sometimes detected at longer wavelengths of UV (Tyrrell, 1991).

Nucleotide excision repair

Nucleotide excision repair is the most extensively studied, universal, and highly conserved DNA repair mechanism, which is used in all cell types. Many types of DNA damage, particularly those produced after UV radiation or exposure to chemical agents, are repaired by NER. As in base excision repair the damaged base or nucleotide is excised and replaced with a newly synthesized DNA strand by using a complementary strand as a template. An enzyme system hydrolyzes two phosphodiester bonds, one at either side of the damage, to generate an oligonucleotide carrying the lesion. After removing the oligonucleotide, the gap is filled by repair synthesis and ligated to the existing strand (reviewed by Frieberg, 1995). Upon UV radiation, DNA strand breaks are formed only in the context of repair, not directly by UV. Rationally enough, NER occurs more efficiently in the leading strand of DNA and in actively transcribed genes (Mellon et al., 1987) . Moreover, some components of TFIIH, a factor essential for transcription initiation, are also required for NER suggesting that repair and transcription are not fully separate processes but are linked to each other and share, at least partly, the same components (Bootsma and Hoeijmakres, 1993). For example, in NER several DNA unwinding enzymes called helicases are needed for damage recognition and removal of a damaged segment, and some of these helicases, ERCC2 and ERCC3, are also components of TFIIH complex thus acting both in nucleotide excision repair and in transcription initiation (Bootsma and Hoeijmakres, 1993).

Three distinct hereditary diseases are associated with defects in nucleotide excision repair: xeroderma pigmentosum (XP), Cockayne´s syndrome (CS), and

trichothiodystrophy (TTD). Although in all syndromes gene(s) involved in NER is/are mutated, the clinical pictures are quite diverse. Contrary to expectations, only XP patients are prone to cancers. They exhibit many sun light-induced diseases, including skin cancer, and some neurological defects (Bootsma, 1993). In fact, discovery of defective NER in XP patients led to subsequent cloning of nuclear excision repair genes, which were named XP genes or ERCCs (excision repair cross complementing). XP patients have been assigned to seven different groups (XP-A through XP-G), each carrying a mutation in a different gene. In Cockayne´s syndrome, the CSB (=ERCC6) and XP-D (=ERCC2) genes are mutated, resulting in a unique repair defect; a lack of the coupling of transcription for repair (van Hoffen et al., 1993). TTD is caused by mutations of at least XP-B (=ERCC3), XP-D (=ERCC2), and XP-G (=ERCC5) genes (Stefanini et al., 1993). Although XP patients have a high incidence of UV-induced malignacies, there is no other evidence of malfunction of NER in cancers, besides this rare syndrome.

RETINOBLASTOMA GENE PRODUCT

Children with familial retinoblastoma carry a germline mutation on one allele of the retinoblastoma gene (RB1), and mutation of the other allele later gives rise to clinical cancer at very young age (Cavenee et al., 1988). It was soon discovered that RB was mutated in several other cancer types as well, and the identification and characterization of the RB gene made it the first tumor suppressor cloned.

RB pathway in cell growth control

Rather than a tumor suppressor only, pRB should be considered as a core element of an essential pathway governing cell cycle progression, differentiation, cell death, and tumorigenesis. This "RB pathway" consists of D-type cyclins and Cdk4 and 6, which are largely responsible for the cell cycle phase-specific phosphorylation and inactivation of pRB, p16 CKI inhibiting the activity of those kinase complexes, pRB itself, and the family of E2F transcription factors regulated by pRB (Fig 2.). The intactness of this pathway seems to be of utmost importance in tumor suppression, since one or more of these cell cycle regulators appear to be aberrant in nearly every tumor (reviewed by Sherr, 1996). Activation of components with oncogenic potential (cyclin-Ds, Cdk4/6, cyclin E, E2Fs), or inactivation of tumor suppressors (CKIs, pRB) may lead to uncontrolled cell proliferation. Depending on cancer type, a variety of different

molecular mechanisms are detected in deregulation of different components of this pathway.

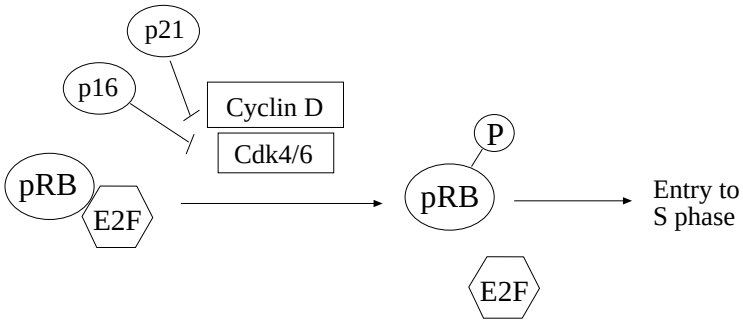

Fig. 2. RB pathway. Cell cycle phase dependent phosphorylation of pRB by cyclins D and Cdk4/6 releases E2F function. p16 and p21Cdk inhibitors are able to inhibit cell cycle progression.

Phosphorylation of pRB

The main task of pRB is to control the progression of the cell cycle through late G1 and the commitment to enter S. pRB is periodically phosphorylated on its serine and threonine residues; at least a dozen phosphorylation sites have been identified (Knudsen and Wang, 1996). Unphosphorylated pRB has to exert its functions during the first two thirds of the G1 phase, during which the cell is sensitive to mitogenic signals. Around and after this restriction point pRB becomes phosphorylated, and is in this form unable to influence the course of the cycle until it is dephosphorylated again after M phase (Ludlow et al., 1990)(Fig.1.). Cyclin D1, 2, and 3 together with Cdk4 and Cdk6 are most prominently involved in the phosphorylation of pRB, but overexpression of cyclin E and A also enhances the phosphorylation of pRB *in vitro* (Hinds et al., 1992). Probably cyclin Ds are responsible for pRB phosphorylation during G1-phase progression, while kinases associated with cyclin E/A take over the phosphorylation of newly synthesized pRB at the G1/S transition and thereafter (Weinberg, 1995; Bartek et al., 1996). Cyclin D1 overexpression often seen in squamous cell carcinomas is mainly due to gene amplification, whereas in mantle cell B lymphomas an increased level of cyclin D1 is caused by cromosomal translocation t(11;14) (Bartek et al., 1997).

The activity of pRB is modulated in response to both positive and negative growth signals: mitogens favoring cell proliferation raise the level of cyclins leading to phosphorylation of pRB, whereas growth inhibitory signals, such as transforming growth factor β (TGF-β) and contact inhibition, modulate the activity of Cdks by CKIs resulting in inhibition of pRB phosphorylation (Reynisdottir et al., 1995). Thus, neither signal types affect pRB directly, but through modulating the function of cyclin-Cdk complexes.

Complex of pRB with E2Fs

pRB exerts its effects by interacting cell cycle phase-dependently with a range of factors regulating proliferation and differentiation. The growth-inhibitory function of hypophosphorylated pRB was thought to be based on its ability to bind and sequester E2F transcription factors (Chellappan et al., 1991)(Fig. 2.). Later, the pRB-E2F complex was found to actively reppress gene transcription (Hamel et al., 1992; Weintraub et al., 1992), and a recent piece of evidence indicates that the active repression by E2F-pRB complex, not the inactivation of E2F, is the prerequisite for pRB-mediated G1 arrest (Zhang et al., 1999). pRB is a member of the "pocket-protein" family, as a large A/B "pocket" consisting of A and B protein-binding domains is responsible for binding E2Fs and many other factors. The E2F family comprises six transcription factors (E2F1-5 and dE2F), which heterodimerize with members of DP family and control the expression of a number of S-phase genes containing E2F-responsive elements in their promoters (reviewed by Dyson, 1998). The growth inhibitory function of pRB is not limited to E2F repression-mediated inhibition of RNA polymerase (pol) II, but pRB can also inhibit the activity of pol I (Cavanaugh et al., 1995) and pol III (White et al., 1996). Moreover, pRB is able to repress the transcription of Myc (Cziepluch et al., 1993) and Fos via retinoblastoma controlling element and to enhance the transcription of insulin-like growth factor II (IGFII).

Excess RB has been reported to protect cells from apoptosis, probably by inhibiting E2F-mediated apoptosis (Haas-Kogan et al., 1995; Haupt et al., 1995; Berry et al., 1996). E2F-1 overexpression drives cells inappropriately into the S phase (Johnson et al., 1993) and causes apoptosis, supposedly in a p53 dependent manner (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). Interestingly, expression of p19ARF, an activator of p53, is induced by E2F-1 providing a link between the pRB and p53 pathways (DeGregori et al., 1997). E2F-1 seems to be a protein of dual nature. On one hand, it behaves like a classic oncogene in transformation assays (Johnson et al., 1994) and its overexpression promotes tumorigenesis in transgenic mice (Pierce et al., 1998). On the other hand, E2F-1 knock-out mice suffer from numerous tumors (Yamasaki et al., 1996). These opposite effects seem to be dependent on the concentration of E2F in a specific manner: high levels promote apoptosis, medium levels allow cell cycle progression, and low levels cause growth arrest. It is particularly the capacity of E2F-1 to induce apoptosis that seems to be relevant for its conditional tumor suppression function (Macleod, 1999).

The inhibition of E2F has turned out to be too simplistic an explanation for the tumor suppressing properties of pRB. In contrast, it has been shown that E2F binding is not sufficient for growth suppression by pRB (Qian et al., 1992). Moreover, amplification

of E2F in cancer cells is extremely rare and has been found only once (Saito et al., 1995). However, tumors in RB +/- mice are smaller and are formed later in the absence of E2F (Yamasaki et al., 1998). To date, pRB has been found to bind to at least 50 different proteins, and clearly some of these interactions may have a significant role in RB-mediated tumor suppression. For example, the C-terminal domain of pRB binds mdm2 (Xiao et al., 1995) and c-Abl tyrosine kinase (Welch and Wang, 1995), both involved in regulation of tumorigenesis. By binding multiple effectors pRB can simultaneously modulate the signals from diverse growth controlling pathways.

The balance between RB and p16

The inverse correlation between pRB and Cdk-inhibitor p16 expression that is observed in many cancer types indicates that they are connected to each other by a negative feedback loop. As a G1 phase specific Cdk-inhibitor, p16 is mainly involved in the suppression of pRB phosphorylation by cyclin D-Cdk4/6 complexes.

One of the first observations indicated that p16 was overexpressed in tumor cells harboring oncoprotein-inactivated pRB (Serrano et al., 1993). Subsequently, the loss of RB function was associated with high levels of p16 in many tumor types, particularly in gliomas and bladder carcinomas. The introduction of wild-type p16 arrests normal cells in late G1, a mechanism requiring functional pRB (Lukas et al., 1995; Serrano et al., 1995). The ability of pRB to negatively regulate p16 promoter explains the high levels of p16 in the absence of functional RB (Li et al., 1994). Conversely, p16 has been shown to induce transcriptional downregulation of RB, completing the feed-back loop (Fang et al., 1998).

Point mutations and small deletions of p16 are common in pancreatic adenocarcinomas, esophageal carcinomas, and biliary tract cancers, and germline mutations of p16 are found in hereditary melanomas and pancreatic cancers (reviewed by Pollock et al., 1996). Other possible inactivating mechanisms include homozygous deletions and methylation of the p16 locus. p16 Cdk-inhibitor is also the most often lost cell cycle regulator in established cell lines; 70% of immortalized cell lines contain no functional p16.

Inactivation of RB

In addition to disturbing RB function by abnormal regulation of its phosphorylation, pRB activity can be prevented by point mutation or deletion of RB gene, or by binding of pRB with viral oncoproteins. The protein products of transforming DNA viruses, such as simian virus 40 (SV40) T antigen (DeCaprio et al., 1988), human papillomavirus (HPV) E7 protein (Dyson et al., 1989), and adenovirus E1A protein

(Whyte et al., 1988) are able to bind to the hypophosphorylated form of pRB and prevent its association with E2F. Elimination of pRB activity is necessary but not sufficient for virus induced transformation, and explains why the same viruses, though through different proteins, also target p53.

Besides being defective in all retinoblastomas, inactive RB alleles are encountered in approximately 90% of small cell lung carcinomas (Harbour et al., 1988; Yokota et al., 1988; Horowitz et al., 1990), in 20-30 % of non-small cell lung cancers (Reismann et al., 1993), and frequently in bladder, pancreatic (Ruggeri et al., 1992), prostate, breast (Lee et al., 1988; T´Ang et al., 1988; Bookstein et al., 1989), and mesenchymal cancers (Friend et al., 1986; Weichselbaum et al., 1988). Patients with familial retinoblastoma are also prone to soft tissue sarcomas and osteosarcomas (DerKinderen et al., 1988). While major deletions are responsible for defective RB in retinoblastomas, inactivating point mutations in the pRB pocket are more common in other cancers. Interestingly, high levels of pRB in bladder cancer has been associated with as poor a prognosis as is loss of pRB, and overexpression of pRB was found to correlate with loss of p16 further confirming the importance of the intactness of the whole RB pathway (Benedict et al., 1999).

RB appears to be indispensable for development, because RB-/- mouse embryos die at midgestation due to defective erythropoiesis and uncoordinated proliferation and cell death in the liver, lens and nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Although RB is clearly important in cellular differentiation, RB knockout mice develop normally until the 13th gestational day, and RB-/- mouse embryo fibroblasts (MEFs) derived from RB-/- mice before this stage are able to proliferate but have a somewhat shorter G1 phase than normal MEFs (Herrera et al., 1996). Other RB family members, p107 and p130, may partly take over the functions of pRB and explain the normal growth at very early stages of development. RB heterozygous mice are viable and develop pituitary and thyroid tumors, but contrary to expectations, exhibit no retinoblastomas (Hu et al., 1994; Nikitin and Lee, 1996). However, RB+/- p107 -/- mice show retinal dysplasia (Lee et al., 1996) or retinoblastoma (Robanus-Maandag et al., 1998) depending on the mouse strain, suggesting that due to overlapping functions inactivation of both pRB and p107 is needed for retinal phenotype. Simultaneous inactivation of pRB and p53 also predisposes to retinal tumors; p53 function would otherwise commit the cells to apoptosis (Howes et al., 1994).

p53 TUMOR SUPPRESSOR PROTEIN

Loss of p53 function in tumor formation

Originally, p53 protein was found to complex with SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), and its overexpression appeared to cause oncogenic transformation of cells. These findings misled the first investigators to regard p53 as a tumor antigen or an oncogene. Later, when the wild-type conformation of p53 was found, the true nature of p53 was revealed. Wild-type p53 was often found inactivated in human tumor cells (Wolf and Rotter, 1985; Baker et al., 1989; Kelman et al., 1989; Nigro et al., 1989) and, when introduced into cells, was growth suppressive rather than oncogenic (Finlay et al., 1989; Baker et al., 1990; Michalowitz et al., 1990).

p53 mutations

p53 function is lost in over 50% of human cancers evidencing the role of p53 in tumorigenesis (Hollstein et al., 1991). A recent database contains over 7500 p53 mutations in all human tumors (Beroud and Soussi, 1998). The mutation frequency varies from one cancer type to another; the average mutation frequencies in most common malignancies are 70% in lung carcinoma, 65% in colon cancer, 45% in stomach cancer, and 30% in breast and prostate cancers (Beroud and Soussi, 1998). Tumor types that rarely contain p53 mutations may still harbor some other changes inactivating p53 pathway. Nevertheless, some cancer types, such as teratocarsinomas, seem to be highly resistant for selecting p53 mutations (Lutzker and Levine, 1996). Most commonly the genetic change comprises a missense mutation in one allele, producing a faulty protein with an increased half life. The vast majority of these p53 mutations are clustered in the DNA-binding domain of the protein, particularly within the four evolutionary conserved regions, the so-called hot spots (Hollstein et al., 1994) (Fig.3). Point mutations can be divided into two classes according to the way they change the DNA-binding ability: "contact" mutants decrease p53 DNA-binding by interfering with those amino acid residues making contact between p53 protein and DNA, whereas "conformational" mutants, making up only 7% of p53 mutations, disrupt the structural elements of the protein. Instead of a specific, common mutant conformation of p53, mutant p53 is more likely a defectively folded form of the protein (Cho et al., 1994).

The mutants of p53 may achieve tumorigenic properties also with a mechanism other than losing the wild-type functions. Some p53 mutants can oligomerize with wild-type p53 and hinder the DNA-binding and transactivation functions of p53 through their dominant-negative effect. For example, p53 wild-type mice with a dominant-negative

transgene developed tumors in which selection has favored mutant p53 over wild-type p53. Moreover, mutant p53 transgene was able to cause tumors in mice harboring one or two alleles of wild-type p53, but not in mice lacking p53, demonstrating the difference between dominant-negative effect and gain-of-function properties (Harvey et al., 1995). Although generally inactive, some mutant forms are still capable of binding DNA and transactivating p53 target genes, at least to a certain degree or in certain circumstances.

While some p53 mutants may mimic the actions of wild-type p53, some wild-type p53 proteins can be functionally inactive. The cytoplasmic location of wild-type p53 sometimes seen in certain rare forms of breast cancer, neuroblastoma (Moll et al., 1995), and melanoma (Weiss et al., 1995) renders p53 incapable of transactivating genes in the nucleus. High levels of mdm2, due to gene amplification or other modifications, may also inactivate wild-type p53 by inhibiting the transactivation capability of p53 and by targeting p53 protein for degradation.

Although inactivation of p53 may be unnecessary or insufficient for tumor initiation, in many cancers it contributes to malignant progression, metastatic potential, and invasiveness (Hsiao et al., 1994). Tumor development requires adequate and continuously growing blood supply, and the involvement of p53 in angiogenesis explains its participation in later stages of tumor progression . Expression of wild-type p53 has been shown to stimulate inhibitors of angiogenesis (Dameron et al., 1994; Van Meir et al., 1994), and a mutant p53 can participate in the stimulation of the angiogenic vascular endothelial growth factor (VEGF) gene (Kieser et al., 1994). In addition, hypoxia, the usual condition in the center of a tumor, induces p53-dependent apoptosis of cells containing wild-type p53, whereas cells harboring mutant p53 survive allowing tumor expansion (Graeber et al., 1996).

p53 mutations in Li-Fraumeni syndrome

Li-Fraumeni syndrome (LFS) provides a human model of nonfunctional p53. The patients have inherited germ-line p53 mutations, which highly predispose them to multiple primary cancers at an early age (Malkin, 1993). The characteristic neoplasms in this dominantly inherited disorder include breast cancer, sarcoma, and glioma, as well as several more rare tumors arising in childhood (Birch et al., 1994). Families with a germline missense mutation in the DNA-binding domain of p53 possess higher cancer rates at earlier age than the families with other type of p53 mutations (Birch et al., 1998). However, heterozygous germ-line p53 mutations have been found in only 71% of LFS families, and many tumors in these patients show no loss of heterozygosity (Sedlacek et al., 1998), questioning the role of p53 as the sole cause of cancer development (Varley et al., 1997).

p53-/- mice and MEFs

p53-/- mice, contrary to RB deficient mice, are viable but are highly prone to both spontaneous and induced tumors, especially lymphomas (approximately 60 % of tumors) and soft tissue sarcomas (20% of tumors) appearing within first 6 months (Donehower et al., 1992; Harvey et al., 1993a). The tumor incidence in heterozygous p53+/- mice is intermediate compared to normal and homozygous p53 -/- mice, and tumor cells of these mice have often lost the remaining wild-type allele. Interestingly, the tumor spectrum in these animals consists predominantly of osteal and soft tissue sarcomas instead of lymphomas (Harvey et al., 1993b). The difference in tumor spectra between heterozygotes and homozygotes may depend on how easily a particular tissue type loses the wild-type allele or how low levels of p53 can still maintain normal p53 functions. The predisposition of p53-/- mice to lymphomas is also of interest, because the loss of p53 is rarely encountered in lymphomas.

p53-/- MEFs derived from p53-deficient mice show several cellular abnormalities: the cells have shortened halflife, they fail to senescence at high passages, and are able to grow at low density (Harvey et al., 1993c) . In addition, chromosomal abnormalities and aneuploidy appear at early passage giving rise to genomic instability. Cells lacking p53 are also highly tolerant to different genotoxic impulses leading to accumulated, unrepaired DNA lesions (Lowe et al., 1993a).

Loss of p53 may sometimes be sufficient for immortalization of cells, but more often a lack of p53 allows other genetic changes favoring tumor formation, for example oncogene activation, to occur. Thus, depending on the original genetic backround as well as on accumulating of other mutations, a considerably different tumor pattern and incidence may arise.

The structure of the p53 protein

p53 is a nuclear transcription factor, which activates the transcription of several target genes involved in the regulation of cell growth and apoptosis. Human p53 protein consists of 393 amino acid residues, which can be roughly divided into four structurally and functionally different domains (Fig. 3.). The acidic amino-terminal domain consisting of the first 42 aa is responsible for the transactivating properties of the protein, without which the induction of target genes cannot occur. Amino acids ranging from 102 to 292 form the central sequence-specific DNA-binding domain (Bargonetti et al., 1993; Pavletich et al., 1993), the most common location of p53 mutations. Between these two domains a novel proline-rich domain localized between aminoacids 64 and 92 has been identified (Walker and Levine, 1996). The carboxy-terminal domain displays

many functions, and can be further divided into the oligomerization domain (amino acids 324-355) and the basic C-terminus with the last 30 amino acids.

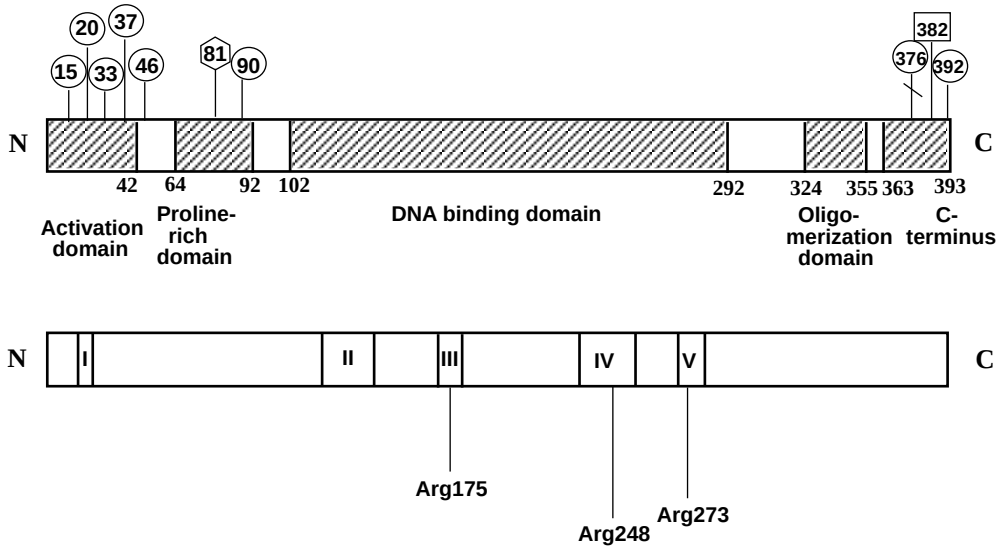

Fig. 3. Structure of human p53 protein. In the upper panel main functional domains and *in vivo* phosphorylation sites of p53 are shown. In contrast to other modification sites, Ser376 should be dephosphorylated and Lys382 acetylated for increased activity of p53. In the lower panel five conserved regions as well as the three most often mutated amino acids are indicated.
◯ = serine, ⬡ = threonine, ▭ = lysine.

Amino-terminal domain

The N-terminal as well as the C-terminal domain of p53 binds to numerous proteins involved in DNA replication and repair. A list of factors binding to and affecting the transactivation domain includes many general transcription factors, such as TATA box-binding protein (TBP) (Horikoshi et al., 1995) and TBP-associated factors (TAFs) TAF70 and TAF31 (Lu and Levine, 1995; Thut et al., 1995), all of which are subunits of the general transcription factor TFIID. By binding to TBP, wild-type p53 can repress genes lacking a p53-binding site. p62, a component of the dual transcription /repair factor TFIIH, and RP-A, a single stranded DNA-binding protein, also interact with amino-terminus of p53. p53 can thus induce growth arrest not only by activating growth suppressive target genes but also by the repression of transcription by sequestering and binding to TBP, RPA and other proteins involved in replication (Seto et al., 1992; Mack et al., 1993). C-Abl is activated after DNA damage and binds to the N-terminal domain of p53 enhancing its transactivation function (Goga et al., 1995), whereas adenovirus E1B 55 kD protein inhibits p53-mediated apoptosis. Most importantly, the negative regulator of p53, mdm2, binds to the N-terminus of p53, sharing the same amino acids 22 and 23 of p53 with E1B 55 kD protein (Lin et al., 1994). In addition, both N- and C-

terminus are subject to diverse phosphorylation modifications that are involved in the regulation of p53; these changes are discussed in the context of p53 regulation.

The proline-rich domain

This domain contains five repeats of the PXXP motif, where P represents proline and X any amino acid (Walker and Levine, 1996). Removal of proline-rich domain does not affect cell cycle arrest but results in an impaired capability of p53 to suppress growth of tumor cells (Walker and Levine, 1996), and impaired apoptotic activity (Venot et al., 1998; Zhu et al., 1999). Interestingly, p53 mutants lacking the proline-rich domain can selectively transactivate certain target genes; natural p21, mdm2, and bax promoters are stimulated while the transactivation of PIG3 is inhibited (Venot et al., 1998). Additionally, this domain seems to be important for transcriptional repression (Venot et al., 1998), which together with selective transactivation is involved in apoptosis mediated by the proline-rich region.

DNA-binding domain

The central sequence-specific DNA-binding domain is an independently folded, Zn^{2+} ion requiring domain. The four conserved regions within the core domain make contact with the major and minor grooves of p53-binding sites, while the less conserved regions serve as a structural element, the ß-sandwich, which helps positioning the residues that interact with DNA (Cho et al., 1994). Proteins defective in DNA binding are incapable of transactivating target genes and transmitting most of the effects of p53. The importance of DNA binding for p53 function is underscored by the fact that p53 residues most often mutated in human cancer, among them Arg248, Arg273, and Arg175, are involved in DNA binding and located in conserved regions (Fig. 3). SV 40 T antigen binds to and blocks the DNA-binding domain, whereas the p53 binding proteins 53BP1 and 53BP2 enhance p53-mediated transcriptional activity (Iwabuchi et al., 1998). Although the tumor suppression function of p53 is highly linked to the core domain, other domains may also be needed for the full suppression of transformation.

The three-dimensional co-crystal structure of the DNA-binding domain (Cho et al., 1994) confirmed the tetrameric nature of p53 molecule. In solution and in wild-type conformation p53 is found as a stable dimer of a dimer, a conformation characteristic but not unique to p53 protein. Four DNA-binding domains together are more effective than one in reaching multiple and sometimes distant p53-binding sites in a target gene promoter (for example in p21 and cyclin G genes) (Zauberman et al., 1995). Tetrameric p53 protein binds to two repeats of a consensus DNA sequence 5´-PuPuPuC(A/T)(T/A)GPyPyPy-3´ (El-Deiry et al., 1992).

The C-terminal domain

A flexible linker connects the DNA-binding domain to tetramerization or oligomerization domain. The structure of this domain has been elucidated by three-dimensional nuclear magnetic resonance spectroscopy (Lee et al., 1994; Clore et al., 1995a; Clore et al., 1995b) revealing a certain homodimerizable motif (ß-sheet-turn-α-helix). Only a small fraction of point mutations in p53 are located in this domain, and some evidence suggests that tumor cells select for an intact oligomerization domain, because tetramerization may be needed for complexing with wild-type p53 and exerting dominant-negative phenotype. Although the role of the oligomerization domain in transformation may be negligible, it is, as well as the activation domain, needed for growth suppression.

The extreme C-terminus is an important and autonomous domain regulating the activation of the whole p53 molecule. It can bind nonspecifically to different forms of DNA-strands (Bakalkin et al., 1994; Lee et al., 1995) and reanneal complementary single strands of DNA and RNA (Brain and Jenkins, 1994; Wu et al., 1995). Normally, the carboxy-terminus acts as an autoinhibitory region, but several factors are able to abolish this negative-regulatory function. p53 function can be activated by C-terminal deletion (Hupp et al., 1992), antibody binding (Hupp et al., 1993; Halazonetis et al., 1993), phosphorylation or other posttranslational modifications (Shaw et al., 1996), or by binding of small C-terminal peptides (Hupp et al., 1995). Monoclonal antibody PAb421 recognising amino acids 370-378 is a well known activator of p53 sequence-specific DNA binding. Besides activating wild-type p53, some of these modifications can reactivate most naturally occurring mutant p53 proteins (reviewed by Selivanova et al., 1998). For example, C-terminal synthetic peptide corresponding to residues 361-382 has been shown to restore the apoptotic and growth suppression functions of at least two common p53 mutations (R248Q and R273H) in human tumor cells (Selivanova et al., 1997). According to the allosteric model, the interaction between the C-terminal regulatory domain and its binding site in p53, probably in the core domain, results in a conformation that is unable to bind DNA. C-terminal modifications are postulated to change the conformation of p53 so that the DNA-binding domain is exposed and sequence-specific binding is allowed. Recently it was demonstrated that the peptide derived from the C-terminal domain of p53 binds to the core domain of mutant p53 (Selivanova et al., 1999). The binding of C-terminal peptide may relieve the inhibition by disrupting the interaction between C-terminus and its binding site by competitive inhibition, and moreover, the peptide may stabilize the DNA-binding domain or establish new DNA contacts (Selivanova et al. 1998).

Short (20-39 nucleotides) single strands of DNA interact with the C-terminus and effectively activate DNA binding of p53, whereas longer and double stranded DNA

inhibit this function (Bakalkin et al., 1994; Jayamaran and Prives, 1995). Thus, the role of the C-terminus in damage recognition and DNA repair is significantly based on its ability to 1) bind single stranded DNA, generated for example during replication errors and excision repair processes, 2) catalyze the reassociation of single stranded DNA into the double stranded form, and 3) interact with DNA helicases. Interestingly, antibody pAb421 that activates the DNA-binding function of p53, can at the same time inhibit the nonspecific binding and reannealing activities (Jayamaran and Prives, 1995; Wu et al., 1995). It has been postulated that two different active conformations of p53 may exist, one being activated by an antibody or phosphorylation and leading to enhanced specific but decreased nonspecific DNA binding, and the other being incapable of DNA binding but remaining active for other functions. Thus, many kinds of modulations in the C- and N-terminus can regulate the p53 molecule between inactive and active, perhaps more than one, conformations.

p53 homologues

Quite recently two p53 homologues, p63 and p73, have been identified, sharing considerable homology with the activation, DNA binding, and oligomerization domains of p53 (Kaghad et al., 1997; Yang et al., 1998). However, p73 is not induced by DNA damage, but its overexpression causes growth arrest and apoptosis (Jost et al., 1997). Both p63 and p73 are able to transcriptionally activate p53 target genes depending on which of the multiple isoforms they exist in (Jost et al., 1997; Yang et al, 1998). A tumor suppressor function has been suggested at least for p73, since neuroblastomas often lacking p53 mutations exhibit deletion of the genomic region containing the p73 gene.

Induction of p53 by physiological and genotoxic stress

Several different cellular and environmental conditions, usually harmful to the cell or organism, cause the accumulation and activation of p53, resulting in growth arrest or apoptosis. Although high p53 levels are detected during development at specific tissues including the central nervous system, p53 protein is otherwise present at a very low level in normal cells. Although the protein is being constantly translated, it is actively and efficiently degraded until needed. The half-life of normal, unactivated p53 protein is less than 30 minutes, whereas posttranslational stabilization increases the time to several hours (Kastan et al., 1991). Additionally, p53 can itself regulate its activity at the translational level. In normal cells translation of p53 mRNA is constitutively inhibited by its 3´ untranslated end. Furthermore, p53 protein can bind to its own mRNA repressing its translation, but upon DNA damage the repression is suppressed

(Mosner et al., 1995). The presence of p53 at extremely low levels in normal conditions and its activation upon damaging or otherwise harmful situations suggest that p53 is not needed during the normal cell cycle, but is called into action when problems arise. This mode of function differs from the action of other tumor suppressors, for example RB, which is constantly working throughout the cycle.

Genotoxic stress, for example UV and γ-irradiation, and cytotoxic drugs, is a strong inducer of p53 (Fig. 4.). Although the extent and kinetics of p53 accumulation and activation differ somewhat among these stimuli, the common denominator is the presence of DNA strand breaks (Nelson and Kastan, 1994). Besides DNA double-strand breaks, which are directly formed by γ-irradiation and indirectly by DNA repair processes after UV radiation and cancer therapy drugs, single strand breaks can also induce p53 (Di Leonardo et al., 1994). Introduction of restriction enzyme nucleases into the nucleus results in an increase in the level of p53 demonstrating that strand breaks are sufficient for activation of p53 (Siegel et al., 1995).

Furthermore, more general stress situations arising from suboptimal growth conditions, such as hypoxia (Graeber et al., 1994), alterations in redox balance (Hainaut and Milner, 1993), heat, starvation, and nucleotide depletion (Linke et al., 1996) induce p53 (Fig. 4.). Since oxidation inhibits and reduction induces DNA binding of p53 (Hainaut and Milner, 1993; Hupp et al., 1993), inactivation of p53 provides a mechanism by which oxygen radicals can promote tumor formation.

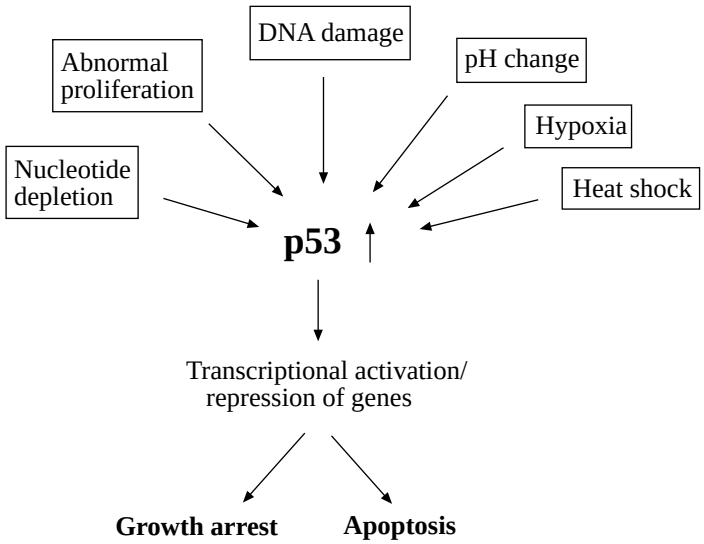

Fig. 4. Inducers of p53 activity. Growth arrest and apoptotic functions of p53 are induced by diverse physiological and genotoxic signals.

In all the above mentioned situations, as a physiological response, p53 is accumulated and activated at the same time, but in other contexts p53 may be accumulated without activation. p53 may complex with viral oncoproteins, for example

SV40 T antigen, leading to an increase in the amount of p53 protein but not in activity. On the other hand, E1A and E7 proteins, which complex with pRB instead of p53, cause p53 stabilization and p53-dependent apoptosis (Lowe and Ruley, 1993; Demers et al., 1994). In addition, p53 mutations couple increased p53 protein level with decreased or absent function. In conclusion, all conditions where p53 is accumulated carry an increased risk for transformation, regardless of the mechanism or reason for accumulation (DNA-damage, infection with tumor viruses or mutation of p53).

Activation mechanisms of p53

Mdm2-mediated degradation of p53

Tight regulation of growth suppressive and apoptotic properties of p53 is essential. Although p53 needs to be rapidly and efficiently activated, it is equally important that p53 response is later adequatly attenuated. It was discovered quite early that p53 was a target of common ubiquitin-dependent proteolysis (Chowdary et al., 1994), and that HPV E6 protein inactivated p53 by targeting it for degradation by the proteasome (Scheffner et al., 1993). Later proteasome inhibitors alone were shown to be able to stabilize and activate p53 (Maki et al., 1996).

Mdm2, itself a target gene of p53 and induced by it, negatively regulates p53 in two ways: by physically interacting with p53 it represses the transcriptional activity of p53 (Oliner et al., 1993; Wu et al., 1993) and mediates its degradation (Haupt et al., 1997; Kubbutat and Vousden, 1997). Mdm2 interacts with p53 N-terminal residues 17-27 (Picksley et al., 1994), which are located in the transactivation domain of p53 and are also subject to a variety of phosphorylation changes. Moreover, this region is one of the highly conserved segments of p53 further highlighting the significance of mdm2 as a regulator of p53. Mdm2 is able to shuttle p53 from the nucleus to cytoplasm (Roth et al., 1998), where the degradation takes place. Additionally, mdm2 may function as an E3 ubiquitin ligase (Honda et al., 1997), whereas others have hypothesised that binding of mdm2 to p300 transcriptional activator/ histone acetylase might be a precondition for degradation (Grossman et al., 1998). Involvement of mdm2 in the degradation of p53 also helps explain why mutant p53 is stable. Mutated, inactive p53 is not able to induce mdm2 and thus degradation via this pathway is blocked leading to increased amounts of defective p53 protein.

Until recently the upstream pathways of p53 between the initiating signals and activation of p53 at the molecular level have been largely speculative, and several different mechanisms for p53 activation have been suggested. At present, the best known activating mechanisms merge into the the same end result: inhibition of mdm2-

mediated suppression of p53. Thus, p53 seems to be activated by releasing it from inhibition.

Phosphorylation of p53 by DNA damage

Posttranslational modification of p53 by phosphorylation of both N- and C-terminal residues has proven to be one of the most important mechanisms for regulating the activity of p53. Phosphorylation of multiple alternative phosphorylation sites in the N-terminus impairs the interaction between p53 and mdm2 and leads to accumulation of p53 (Shieh et al., 1997) (Fig. 5.). Although several kinase and phosphorylation site candidates have been presented, their physiological role still requires clarification. While casein kinase I (CKI), DNA-dependent protein kinase (DNA-PK), mitogen activated protein kinase (MAP), and Jun amino-terminal kinase (JNK) are able to phosphorylate many N-terminal amino acids *in vitro* (reviewed by Steegenga et al., 1996), phosphatidylinositol 3 (PIK-3) like kinases ATM, ATR and DNA-PK have more recently awaken interest as kinases phosphorylating several N-terminal serines *in vivo*.

Kinase mutated in ataxia telangiectasia (ATM) has been demonstrated to possess a central role in the posttranslational activation of p53 after ionizing radiation (Banin et al., 1998; Canman et al., 1998). AT patients suffer from increased cancer risk and show hypersensitivity to ionizing radiation (Savitsky et al., 1995). γ-radiated AT cells with defective ATM show only a minimal increase in the amount and activity of p53, indicating that ATM functions upstream of the p53 signaling pathway (Kastan et al., 1992). ATM, which has a substantial similarity to other proteins in the PI3-K family, has been shown to phosphorylate serine 15 of p53 after γ-irradiation (Banin et al., 1998; Canman et al., 1998). Interestingly, ATM kinase activity is increased only after ionizing radiation, because cells lacking ATM show normal p53-dependent responses (Khanna and Lavin, 1993) and Ser15 phosphorylation (Siliciano et al., 1997) after UV radiation. Ionizing radiation may induce co-factors that differ from those activated after UV-radiation or, alternatively, direct double strand breaks are needed for enhanced ATM kinase activity. Ser15 is probably phosphorylated after UV radiation by ATR, an ATM related kinase (Tibbets et al., 1999).

DNA-PK, an other member of the same kinase family, is known to phosphorylate p53 on Ser15 and Ser37 leading to stabilization and inhibition of p53 degradation by mdm2 (Shieh et al., 1997). Mice with severe immunodeficiency (SCID), which were believed to have no DNA-PK activity, were found, however, to respond to DNA damage normally in a p53-dependent manner (Gurley and Kemp, 1996; Araki et al., 1997). Later it was specified that cells from these animals have retained some DNA-PK activity explaining the normal p53 functions. When true DNA-PK deficient mice were developed, DNA damage was found to accumulate but not activate p53, supporting the

role of DNA-PK in p53 responses (Woo et al., 1998). Although DNA-PK activity is needed for p53 activation upon ionizing irradiation, it is not sufficient alone (Woo et al., 1998). Since Ser15 is phosphorylated by, at least, two different kinases, DNA-damage induced phosphorylation of this residue has been sugggested to be essential for p53 function. Ser15 lies at the N-terminal border of the mdm2-binding site in p53 and phosphorylation of it weakens both the association of p53 with mdm2 and the repression of p53 by mdm2 (Shieh et al., 1997). In addition to phosphorylation of Ser15, Ser33 and Ser37 are also phosphorylated by both UV and ionizing irradiation (Shieh et al., 1997; Siliciano et al., 1997; Sakaguchi et al., 1998). At present, a constant flow of novel N-terminal phosphorylation sites are emerging, exemplified by Ser20 (Shieh et al., 1999; Unger et al., 1999), Ser46, Thr81, and Ser91; all these sites are phosphorylated by DNA damage.

The C-terminus of p53 is also modified by cellular stress (Fig. 3.). An expanding list of protein kinases have been shown, notably *in vitro*, to phosphorylate p53 at different C-terminal residues: protein kinase C (PKC) at Ser378 (Takenaka et al., 1995), CKII at Ser392 (Herrmann et al., 1991), and Cdk2 at Ser315 (Addison et al., 1990; Bischoff et al., 1990) (Fig.3.). UV but not γ-irradiation induces phosphorylation of human Ser392 representing the only UV specific effect on p53 *in vivo* (Kapoor and Lozano, 1998; Lu et al., 1998). Dephosphorylation of some residues, for example Ser376 after ionizing radiation, appears to be as important for the activation of p53 as is phosphorylation of other residues (Waterman et al., 1998). Moreover, DNA damage has been reported to induce acetylation of C-terminal lysine 382, which has been suggested to reveal the autoinhibition by the C-terminus together with phosphorylation of PK-C and CKII sites (Gu and Roeder, 1997; Sakaguchi et al., 1998). However important phosphorylation of p53 may seem, two recent reports have demonstrated that phosphorylation may not be absolutely required for p53 activity or accumulation after DNA damage (Blattner et al., 1999; Ashcroft et al., 1999). Although nearly all known phosphorylation sites in the N- and C-terminus were mutated, p53 stabilization by UV, gamma irradiation and actinomycin D was not impaired (Blattner et al., 1999), and the mutant p53 proteins retained normal stabilization and transactivation functions upon UV treatment (Ashcroft et al., 1999).

Activation of p53 by p14^{ARF}

A different p53 activation model has been presented in the form of p14^{ARF}, ARF standing for alternative reading frame. A single genetic locus, *INK4a/ARF*, encodes two different proteins, p16^{INK4a} Cdk-inhibitor and p14^{ARF} (Quelle et al., 1995a). p16^{INK4a} protein is encoded by three exons, 1α, 2, and 3, of which exons 2 and 3 are shared by p14^{ARF} but read in a different reading frame. In addition, exon 1α of p16 is replaced by

exon 1ß in $p14^{ARF}$, resulting in two totally different proteins with no amino acid similarities (Mao et al., 1995; Quelle et al., 1995a; Stone et al., 1995). The ARF protein, of size 19 kD in mouse and 14 kD in human, induces G1- and G2-phase growth arrest when introduced into a variety of different cells. However, this activity depends on intact p53 (Quelle et al., 1995b; Stott et al., 1998). Ectopic ARF expression leads to stabilization of p53 and induction of its target gene transcription (Kamijo et al., 1997). The dependency of ARF on p53 was evidenced by experiments where transfection of p53-positive but ARF-negative NIH3T3 cells with ARF led to p53-dependent induction of p21(Kamijo et al., 1998). However, ARF -/- cells overexpressing p53 were not able to induce p21, indicating that an increase in the level of p53 alone in this setting was not sufficient for p21 induction, and demonstrating that ARF acts upstream of p53 (Kamijo et al., 1998). Additionally, in ARF-/- cells p53 activation was normal after DNA damage (Kamijo et al., 1997). Thus, while ARF function is dependent on functional p53, p53 action can be activated without ARF, suggesting that different p53 activating signals utilize independent signaling pathways.

Different proliferative signals, such as c-myc (Zindy et al., 1998), E1A (de Stanchina et al., 1998) and E2F (Bates et al., 1998) lead in normal fibroblasts to ARF activation and p53-dependent apoptosis. These proteins can activate p53 also ARF-independently, but much higher concentrations of oncoproteins are required in the absence of ARF. The underlying mechanism of p53 activation by ARF involves inhibition of mdm2 function. The N-terminus of ARF interacts with the C-terminus of mdm2, leading to repression of the mdm2-dependent suppression of p53 (Pomerantz et al., 1998; Zhang et al., 1998). ARF, which is localized in nucleoli (Quelle et al., 1995a; Pomerantz et al., 1998), inhibits the nucleo-cytoplasmic shuttling of the mdm2-p53 complex and prevents the degradation of both p53 and mdm2 (Roth et al., 1998; Zhang and Xiong, 1999 (Fig.5.). Moreover, ARF may inhibit ubiquitin ligase function of mdm2 (Honda and Yasuda, 1999). The human $p14^{ARF}$ appears not to interact directly with p53 (Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998), but in mouse some evidence has been obtained about the physical interaction between p53 and $p19^{ARF}$ without mdm2 (Kamijo et al., 1998).

After developing ARF knockout mice, it was soon discovered that ARF-/- MEFs were immortalized (Kamijo et al., 1997). Furthermore, like many established cell lines, the ARF-/- cells were easily transformed and immortalized by oncogenic ras, in contrast to normal MEFs in which overexpression of ras leads to a growth arrest (Kamijo et al., 1997). p16 knockout mice lacking exons 2 and 3 were created before ARF was discovered, and numerous tumors in these animals and the aberrant growth of the MEFs derived therein were thought to result from the absence of p16 (Serrano et al., 1996). Surprisingly, when true $p19^{ARF}$ knockout mice, lacking only exon 1ß were developed,

the tumor spectrum and incidence were identical to that of "p16-/-" mice, and tumor cells were shown to express normal p16 (Kamijo et al., 1997). These observations support the role of ARF as a tumor suppressor and may question the role of p16 in tumorigenesis until a knockout mice with loss of only p16 expression are developed.

According to the knowledge gained so far, p53 seems to be crucial in its own regulation. p53 upregulates its inhibitor mdm2, and downregulates its activator ARF (Stott et al., 1998) to keep the inhibitory and stimulatory signals in balance and the p53 level low. As if the activation models are separated and independent they seem ultimately to inhibit or weaken the mdm2-p53 interaction and mdm2-dependent degradation of p53: DNA damage-associated phosphorylation of p53 (ATM/DNA-PK activation pathway) attenuates the interaction between p53 and mdm2, while oncogene activated ARF binds to mdm2 and inhibits destruction of p53, both leading to stabilization and activation of p53 (Fig. 5.).

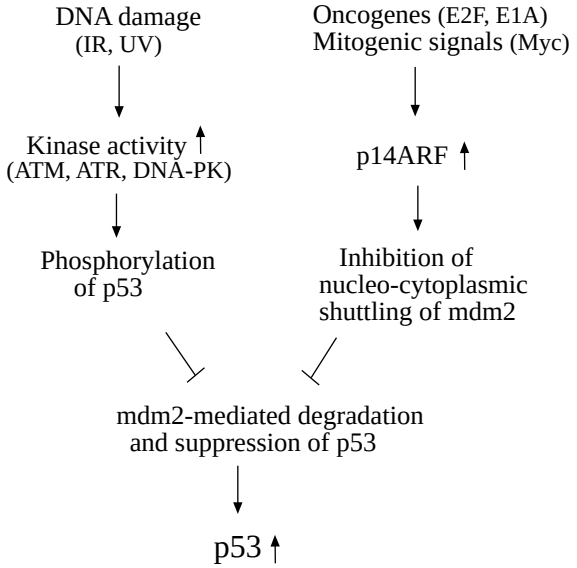

Fig. 5. Regulation of p53 by pathways involving kinase and p14ARF activity. Despite having different activating signals both pathways result in inhibition of mdm2-mediated repression of p53 leading to stabilization and activation of p53.
→ = activation, ⊣ = inhibition.

Multiple effects of p53

The p53 tumor suppressor protein limits cell proliferation and inhibits tumor formation by two main mechanisms: by inducing growth arrest and/or apoptosis (Fig. 4.). The organism is protected from accumulating genetical changes by halting the propagation of harmful mutations to daughter cells. This is achieved by either arresting the cell cycle and allowing time for DNA repair, or by committing suicide by

programmed cell death. In addition to growth and tumor suppression functions, p53 is involved in development and differentiation processes.

Growth arrest

The best characterized antiproliferative function of p53 is its role in G1-phase growth arrest. γ-irradiation of cells rapidly and efficiently leads to p53-dependent G1-phase arrest (Kastan et al., 1992; Kuerbitz et al., 1992). Activation of p53 induces the expression of several target genes, especially p21 Cdk-inhibitor gene (El-Deiry et al., 1993). Upregulation of p21, a potent inhibitor of all Cdks, blocks the catalytic activity of the cyclin-Cdk complex preventing the phosphorylation of many regulatory proteins and leading to inhibition of cell cycle progression (Dulic et al., 1994; El-Deiry et al., 1994). Among the best known consequences is the maintenance of pRB in the underphosphorylated form binding transcription factor E2F-1 (Demers et al., 1994; Slebos et al., 1994). The pRB-E2F complex represses the gene transcription inhibiting the expression of numerous proteins needed in the progression of the S phase. Partly contradictory data have been presented concerning the need of pRB-E2F pathway in p53-mediated growth arrest. In experiments where pRB functions were blocked by viral oncoproteins like the E1A protein of adenovirus, the T antigen of SV40 or E7 of human papillomavirus, p53-dependent growth arrest was inhibited (Demers et al., 1994; Slebos et al., 1994). On the other hand, cells from RB null mice were shown to be capable of undergoing full G1 arrest after γ-irradiation. Several aspects should be taken into account here: first, oncoproteins effect many other cell cycle proteins besides pRB, and secondly, RB null cells have intact RB family members p107 and p130 which may partly overtake the functions of pRB. However, in the absence of clear evidence of competing mechanisms, G1-growth arrest is generally concluded to be exerted via the p21-pRB-E2F pathway.

The role of p21 in p53-mediated G1 growth arrest is evidenced by the observation that overexpression of p21 itself leads to arrested growth (Harper et al., 1995). However, p21-/- cells are only partially defective in γ-radiation induced growth arrest (Brugarolas et al., 1995; Deng et al., 1995) whereas p53 deficiency totally abolishes it, suggesting that other p53 activated factors, for example GADD45, may be also involved. Besides well characterised G1 growth arrest, p53 has a definite role in mediating G2 growth arrest as well (Agarwal et al., 1995; Stewart et al., 1995).

Even in the absence of damaging stimulus p53 functions as a cell cycle checkpoint controller, since p53-/- MEFs exhibit disturbed cell cycle phase distributions compared to wild-type p53 cells (Harvey et al., 1993). Furthermore, the same cells treated with mitotic spindle inhibitors failed to be arrested in the G2 phase, but continued DNA synthesis inappropriately resulting in aneuploidy (Cross et al., 1995). This suggests that

p53 functions as a mitotic spindle checkpoint factor ensuring that replication is not initiated without completion of proper chromosome segregation. Studies with gas1 gene have suggested participation of p53 even in the G0 checkpoint. Gas1 gene, expressing a protein needed to keep cells in G0 state, is not a target gene of p53, but is dependent on wild-type p53 for its function. p53 mutants incapable of transactivation are still able to assist gas1 to arrest cells in G0, giving an example of a p53 checkpoint function devoid of any transactivation property (Del Sal et al., 1995).

Apoptosis

Apoptosis, or programmed cell death, is an efficient means of getting rid of harmful cellular material. Convincing number of experiments have demonstrated a role for p53 in triggering apoptosis in a variety of different conditions. DNA damaging agents, such as ionizing and UV radiation and cytotoxic drugs, c-myc (Wagner et al., 1994) and adenovirus EIA expression (Lowe and Ruley, 1993), and withdrawal of growth factors (Johnson et al., 1993), may result in p53-dependent apoptosis. γ-irradiation of mouse thymocytes provides a classic example of p53-induced cell death (Lowe et al., 1993b). Radiated thymocytes from p53 -/- mice do not undergo apoptosis as do their p53 positive counterparts (Lowe et al., 1993b). Since p53-mediated apoptosis can be blocked by treating myeloid cells with interleukin-6 (Levy et al., 1993) or erythroid cells with erythropoietin, p53 may in these circumstances function as sensor of environmental conditions and growth factor supply. Cancer threapy is also taking advatage of apoptotic pathway, since cytotoxic drugs and irradiation therapy are largely based on the wild-type p53-induced apoptosis. It should be remembered, however, that p53-independent apoptosis, such as cell death after glucocorticoid treatment (Clarke et al., 1993), probably occurs as often as p53-dependent cell death.

p53 participates in the induction of apoptosis directly by inducing the target genes bax, insulin-like growth factor-binding protein 3 (IGF-BP3) (Buckbinder et al., 1995), fas/APO1 (Owen-Schaub et al., 1995), and Killer/DR5 (Wu et al., 1997). Bax, an apoptosis promoter, can form a complex with Bcl-2, a protector against apoptosis, and the balance between these two, among other factors, determines whether apoptosis is induced (Miyashita and Reed, 1995). The IGF-BP3 gene is upregulated by p53 in response to genetic insult (Buckbinder et al., 1995). By binding to IGF it inhibits both survival and mitogenic signals representing an antimitogenic function of p53.

In contrast to the growth suppressive function of p53 that seems to be dependent on the transcriptional activity, apoptotic activity has been difficult to uniformly attribute to any certain domain of the protein. Although p53 induces several genes promoting apoptosis, p53-dependent apoptosis after DNA damage has been detected both in the presence of the translational inhibitor cycloheximide (Wagner et al., 1994) and the

transcriptional inhibitor actinomycin D (Caelles et al., 1994) suggesting that the apoptotic pathway may be independent of the transcriptional activity of p53. The same conclusion was obtained in HeLa cells transfected with mutant $p53_{gln22, ser23}$. This mutation which abrogates the transcriptional activity of p53 was still able to cause p53-dependent apoptosis (Haupt et al., 1995; Yonish-Rouach et al., 1995). Accordingly, neither Bax nor fas/APO1 expression were required for p53-dependent apoptosis *in vivo* (Knudson et al., 1995; Fuchs et al., 1997). In contrast, BRK cells stably expressing E1A and temperature-sensitive $p53_{val135}$ underwent apoptosis when p53 was in its wild-type conformation, but not when the mutant status was applied (Sabbatini et al., 1995a). These controversial findings suggest the existence of two separate apoptotic functions of p53, one depending on the activation of p53 target genes, and the other using direct protein signaling, depending on the cell type or experimental situation. Repression of certain genes may offer one mechanism for transcription-independent apoptosis, since bcl-2 and adenovirus E1B 19 kD protein blocking the p53-dependent apoptosis do not interfere with transcriptional activity but suppress the repression function (Shen and Shenk, 1994; Sabbatini et al., 1995b). This is supported by observation that lack of proline-rich domain of p53 that results in defective apoptosis also impairs the repression function of p53 (Venot et al., 1998). Finally, the large number of proteins interacting with and binding to p53 give rise to numerous possible mechanisms by which p53 may induce apoptosis independent of transactivation.

Growth arrest or apoptosis?

DNA-damaging stimuli and stressful environmental conditions lead to either growth arrest of cells or apoptosis. Several factors determine which way the cells follow. One of the most important factors is the cell type: in mortal primary fibroblasts DNA-damage often leads to transient growth arrest, whereas immortal and hematopoietic cells often react by programmed cell death (Midgley et al., 1995; Haupt et al., 1996).

In addition to DNA-damaging situation, limited availability of growth factors or activation of cellular or viral oncogenes favor the induction of apoptosis rather than growth arrest, in order to eliminate cells with unstable genomes. Different viral proteins have diverse and partially controversial effects on p53 and cell survival. Expression of adenovirus E1A protein in murine fibroblasts stabilize and activate p53 resulting in apoptosis (Debbas and White, 1993). On the other hand, 55 kD E1B protein binds to the N-terminus of p53 and inhibits its transcriptional activity, whereas 19 kD E1B protein acts like bcl-2 blocking the apoptosis downstream of p53 activation (Debbas and White, 1993). Human papillomavirus E7 protein induces p53-dependent apoptosis, but E6 binds to p53 and targets it for proteosome degradation (White et al., 1994). Both apoptosis promoting oncoproteins E1A and E7 share the ability to bind to and inactivate

pRB, not p53, leading to unregulated functions and increasing amounts of E2F-1. Thus, in the presence of intact p53 but in the absence of functional pRB, the apoptotic pathway rather than growth arrest is triggered by these viral oncoproteins.

p53 in DNA repair

As the role of p53 in growth arrest and apoptosis is widely accepted, the involvement of p53 in DNA repair is far from clear. Studies for and against a role of p53 in DNA replication and repair exist. Interaction with many replication and repair proteins, for example RP-A (Dutta et al., 1993), and components of the dual transcription-repair factor TFIIH, including DNA helicases ERCC2 and ERCC3 (Wang et al., 1995; Leveillard et al., 1996), has been the first line of evidence for p53 in these functions. The binding of p53 inhibits the helicase activity of these proteins, as has been shown also with other cellular and viral helicases (Wang et al., 1996). Indirectly, upon stimulation of p21, which complexes with proliferating cell nuclear antigen (PCNA), p53 is involved in the inhibition of DNA replication after DNA damage without interfering with the repair processes (Shivji et al., 1994; Li et al., 1996).

Although in some studies a correlation has been found between p53 deficiency and reduced repair, in others this has not been the case. UV-radiated Li-Fraumeni cells carrying homozygous deletion of p53 exhibited disturbed removal of pyrimidine dimers and photoproducts but normal transcription-coupled repair (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997). However, point mutations accumulated equally (Nishino et al., 1995; Sands et al., 1995), and the rate of NER was identical in both normal and p53-/- mouse cells (Ishizaki et al., 1994) giving evidence against direct p53 functioning in DNA repair. Nevertheless, the loss of p53 reduced the rate and efficiency of nucleotide excision repair in human cells in several experimental conditions (Smith et al., 1995; Wang et al., 1995). One can not either ignore the fact that p53 binds efficiently to damaged DNA, both mismatched and irradiated DNA (Lee et al., 1995; Reed et al., 1995), and that 3´-5´ exonuclease activity of p53 may be used in DNA recombination, replication or repair processes (Mummenbrauer et al., 1996).

Development

Although p53 -/- mice first appeared to develop normally (Donehower et al., 1992), more careful examination of the phenotype revealed developmental disturbances, particularly defects in neural tube closure. For example, at the age of 13.5 days 16% of p53 -/- embryos, all female, showed markable anencephaly (Armstrong et al., 1995; Sah et al., 1995). Additionally, a small fraction of p53 knockout embryos died prematurely, and the embryos of p53-/- pregnant mice were more vulnerable to diverse teratogenes compared to normal mice (Nicol et al., 1995). Mdm2 -/- mice are embryonically lethal,

but the absence of p53 in double knockout mice is able to rescue the embryos (Jones et al., 1995; Montes de Oca Luna et al., 1995). This suggests that in the presence of p53 mdm2 is needed during embryogenesis to negatively regulate p53. Thus, contrary to what was originally believed, p53, in co-operation with mdm2, is not dispensable in early development. p53 is also involved in certain differentiation processes, like B-cell maturation (Aloni-Grinstein et al., 1993) and spermatogenesis (Schwartz et al., 1995).

Transcriptional target genes of p53

The main function of p53 is the transcriptional activation of its target genes. Numerous genes have been presented as candidates for effector genes of p53, but not all of them are able to fulfill the criteria. Even if a p53 binding site is found on a gene or promoter, it should be recognized by wild-type but not mutant p53, and basal transcription of a gene should be increased p53-dependently. Besides containing a p53 response element, a gene should be induced upon cellular stress in a p53 dependent manner. Over the years, some candidates have consolidated their positions as p53-response genes and have shown particular importance in the physiological functions of p53.

p21$^{WAF1/Cip1}$

Besides being the first p53 target gene discovered, p21 is among the most important effector genes. As a universal Cdk-inhibitor, p21 has a central role in p53-induced growth arrest. p21 functions are discussed thoroughly in a separate chapter.

mdm2

Originally mdm2 (murine double minute gene 2) was described as one of the genes amplified in the double minute chromosomes in spontaneously transformed 3T3 cells (Cahilly-Snyder et al., 1987), and later it was found to possess oncogenic potential (Fakharzadeh et al., 1991). Mouse mdm2 gene contains two promoters and numerous exons spread over a large area. One promoter is located upstream of the gene and used for constitutive expression, while the other is present in the first intron and regulated by p53 through two p53 binding sites (Juven et al., 1993; Wu et al., 1993; Barak et al., 1994; Zauberman et al., 1995). Basal expression of mdm2 from its constitutive promoter P1 is thought to be responsible for maintaining low levels of p53, while p53-inducible mdm2 promoter P2 is not activated after DNA damage until interaction between mdm2 and p53 has been disrupted and activation of the target genes has occured. In order to give p53 time to act before it is attenuated by mdm2, transcriptional

activation of mdm2 should occur later than activation of other target genes, or, alternatively, the complex formation between p53 and mdm2 can be retarded.

Mdm2 oncogene encodes several proteins, and the largest 90 kD protein binds to and inactivates p53 (Momand et al., 1992; Haupt et al., 1997; Kubbutat et al., 1997). Multiple other mdm2 proteins have also been detected, and these proteins arise through alternative splicing, proteolytic processing, or posttranslational modifications (Olson et al., 1993). p76, which is unable to bind p53 but binds ARF, is expressed and induced by UV radiation p53 independently (Saucedo et al., 1999). UV radiation has been shown to induce mdm2 in a p53 and UV-dose dependent manner: low UV doses induce the gene rapidly, while at high doses a delay is observed between mdm2 induction and p53 stabilization (Perry et al., 1993).

The mdm2 gene is amplified in about 20% of soft tissue sarcomas (Oliner et al., 1992; Leach et al., 1993) and quite frequently in esophageal tumors, astrocytomas and osteosarcomas (reviewed by Momand and Zambetti, 1997). In addition to gene amplification high levels of mdm2 may result from enhanced translation or other modifications (Landers et al., 1994). Although p53 is mutated in half of human cancers, p53 mutation is rarely found together with mdm2 amplification (Florenes et al., 1994a). Instead of p53 mutation, exceptionally high levels of wild-type p53 are observed in some cancers. Transformed epithelial cells expressing high basal level of wild-type p53 were found to overexpress mdm2 without gene amplification, and with no increase in transcription of other p53 effectors (Blaydes et al., 1997). Additionally, only a small proportion of p53 was found in a complex with mdm2, so that the amount of functional p53 was not totally abolished but was lowered to a reasonable, more physiological level (Blaydes et al., 1997). This suggests that overexpression of mdm2 provides a mechanism by which high wild-type p53 levels can be tolerated without growth arrest or the apoptosis of cells.

The main contribution of mdm2 overexpression to cancer formation is believed to be through elimination of the tumor suppressor function of p53. However, the N-terminal domain of mdm2 is able to activate E2F-1 transcription factor (Martin et al., 1995), and to bind to and inactivate pRB (Xiao et al., 1995). These functions may explain how mdm2 promotes transformation also in cells lacking p53 (Lundgren et al., 1997).

GADD45

GADD45 is the best characterized member of the growth arrest and DNA damage inducible gene (GADD) family. These genes are induced by DNA-damaging agents and some growth arrest conditions, for example nutrient depletion or hypoxia (Fornace et al., 1989). GADD45 is a nuclear protein, the levels of which oscillate slightly during the cell cycle being highest in G1 and lowest during the S phase (Kearsey et al., 1995).

GADD45 can be transcriptionally activated by p53 via p53 consensus binding site at its third intron (Hollander et al., 1993). Although induction of GADD45 upon γ-irradiation is strictly dependent on p53 (Kastan et al., 1992), p53 independent induction of GADD45 is often seen after base-damaging agents, such as UV radiation (Kearsey et al., 1995). Even if p53 may not be necessarily required for the induction of GADD45, disruption of p53 function may reduce the extent of induction (Zhan et al., 1996). An increased level of GADD45, either due to DNA damage or overexpression of the protein, leads to G1 growth arrest of cells (Zhan et al., 1994). A role for GADD45 in DNA repair and inhibition of apoptosis has been suggested by experiments where antisense GADD45 expression decreased DNA repair and sensitized cells to UV- and cisplatin-induced apoptosis, the latter function being opposite to that of p53 (Smith et al., 1996). Moreover, in some experiments GADD45 has been shown to interact with PCNA, and, like p21, inhibit PCNA-dependent replication and thus facilitate nucleotide excision repair (Smith et al., 1994; Hall et al., 1995). However, other studies have not been able to demonstrate the effect of GADD45 on NER (Kearsey et al., 1995).

bax

Bax is a target gene of p53 involved in apoptotic function of p53. Bax forms heterodimeric complexes with bcl-2 and by dominant-negative effect antagonizes its ability to block apoptosis. Interestingly enough, also Bcl-2 seems to be under the control of p53, because p53 can downregulate bcl-2 by repressing its transcription (Haldar et al., 1994; Miyashita et al., 1994).

Others

Cyclin G encodes a cyclin of unknown function (Okamoto and Beach, 1994), which is induced in a p53 dependent manner after DNA damage (Miyashita and Reed, 1995); no Cdk has been yet found to associate with it. The regulation of PCNA by p53 has been questioned, but it seems that transactivation of PCNA is restricted to low concentrations of p53 (Shivakumar et al., 1995; Morris et al., 1996). Additionally, numerous other p53 response genes have been suggested, but the real *in vivo* importance of these target gene candidates needs to be clarified.

Transcriptional repression by p53

Besides being a transcriptional activator, p53 may function as a transcriptional repressor as well, providing yet another mechanism to influence cellular functions. Genes lacking p53-binding site but containing the TATA box can be subject to inhibition by p53 (Mack et al., 1993). c-fos, c-jun, RB, bcl-2, IL-6 and heat shock protein (hsp) 70 are examples of genes repressed by p53. The repressor function of p53

is believed to be especially important in suppression of tumorigenesis and induction of apoptosis.

p21 CDK-INHIBITOR

The $p21^{WAF1/CIP1}$ cyclin kinase inhibitor cDNA was cloned independently by four different groups and thus was given several different names characterizing its functions. Using a yeast-two hybrid system, p21 was reported to bind to Cdk and was named CIP1 (Cdk inhibiting protein 1) (Harper et al., 1993), and, at the same time, it was identified as a gene product activated by p53, called WAF1 (wild-type p53-activated factor 1) (El-Deiry et al., 1993). Later, when DNA synthesis inhibitors were looked for from senescent cells, p21 was cloned as SDI1 (senescent cell-derived inhibitor 1) (Noda et al., 1994). Finally, p21 was identified as MDA-6, melanoma differentiation associated protein, because of its increased expression in melanoma cells after induced differentiation (Jiang et al., 1994).

Cellular effects of p21

Growth arrest

The main effect of p21 seems to be the inhibition of cell proliferation. As a common cyclin-kinase inhibitor, p21 effectively inhibits the kinase activity of Cdk2, Cdk3, Cdk4 and Cdk6, less so Cdk5 and not at all Cdk7 or Cdc2 (Harper et al., 1995). As a result, many regulatory proteins needed for progression of the cell cycle remain unphosphorylated and the cell cycle stops. Originally, p21 was assumed to induce growth arrest only at the G1/S-phase transition, but recently it has been shown to mediate G2 arrest as well (Dulic et al., 1998; Niculescu et al., 1998). p21 may induce growth arrest by at least two different mechanisms: by inhibiting the kinase activity of Cdks and by preventing the actions of PCNA. Two different domains of p21 are responsible for these inhibitory functions (Chen et al., 1995; Luo et al., 1995). The N-terminal part of p21 is required for the Cdk inhibition, while the carboxy-terminal domain interacts with PCNA and prevents the interaction of PCNA with other components of the polymerase assembly. The association with PCNA is a unique feature of p21 that distinguishes it from other CKIs. As an auxillary factor for DNA polymerases ε and δ, PCNA is essential for both DNA replication and DNA repair. Although inhibiting the PCNA-dependent replication and mismatch repair *in vitro* (Flores-Rozas et al., 1994; Li et al., 1994; Waga et al., 1994), p21 appears not to inhibit the function of PCNA in nucleotide excision repair (Shivji et al., 1994; Li et al., 1996).

In normal human fibroblasts, p21 is found in quaternary complexes with cyclin, Cdk and PCNA (reviewed by Kelman, 1997). Cdk in this complex may be inactive or active depending on the amount and stoichiometry of p21 protein. At low or intermediate concentrations p21 seems to function as an assembly factor enforcing the assembly of cyclin D and Cdk4 even by 35-fold and targeting the cyclin/Cdk complex to the nucleus, whereas at high concentrations p21 works as an inhibitor of kinase activity (LaBaer et al., 1997). In a recent study, the antiproliferative functions of p21, inhibition of cyclin/Cdk complexes and inhibition of PCNA, were shown to be independent of each other, the latter function alone being sufficient to block cell cycle progression at the G1/S and G2/M transitions (Cayrol et al., 1998). Interaction of p21 with GADD45 damage response protein is probably also involved in DNA damage checkpoints (Kearsey et al., 1995).

The role of p21 in DNA damage checkpoints has been studied with mice and human cells with homozygous deletion of p21 gene. Gamma-irradiated p21-/- MEFs were partially defective in G1 checkpoint control and in response to double-stranded DNA lesions (Brugarolas et al., 1995; Deng et al., 1995). However, in human colon carcinoma cells HCT116, p21 was absolutely required for p53-mediated G1 growth arrest by ionizing irradiation (Waldman et al., 1995).

Quite opposite to growth arrest, mitogenic stimuli have ben observed to induce p21 (Macleod et al., 1995). Enhanced expression of antiproliferative p21 may be perhaps seen as a compensatory mechanism to prevent the overshoots of mitogenic activation.

Terminal differentiation and senescence

p21 is often induced in the process of terminal differentiation during which cells need to exit from the cell cycle. For example, skeletal muscle cell differentiation correlates with MyoD-induced p21 expression (Halevy et al., 1995). Ectopic expression of p21 has been shown to promote differentiation of hematopoietic cells, such as megakaryoblastic leukemia cells CMK (Matsumura et al., 1997) and myelomonocytic cell line U937 by vitamin D_3 (Liu et al., 1996). Accordingly, several growth factors are able to induce p21 expression; specific examples of the regulation of p21 by some of these are mentioned below.

In addition to being discovered to be an inhibitor of DNA synthesis in senescent cells, disruption of p21 function in normal fibroblasts has been observed to lead to a bypass of senescence and an extended life span of cells (Brown et al., 1997).

Apoptosis and tumorigenesis

Although p21 has been considered inert in apoptosis, a continuous line of evidence suggests that p21 may protect cells from apoptosis (Polyak et al., 1996; Gorospe et al.,

1997; Bissonnette and Hunting, 1998), while in some studies p21 has been shown to promote it (Duttaroy et al., 1997). In contrast to tumor suppressive Cdk-inhibitor p16 and $p14^{ARF}$, p21 appears not to have any role in tumorigenesis. Firstly, p21 mutations are almost never found in human cancers and secondly, p21 knockout mice are not prone to tumors (Deng et al., 1995).

p53-dependent and -independent regulation of p21

Regulation of p21 protein involves both transcriptional and posttranscriptional mechanisms. Regulation at the transcriptional level may be either p53-dependent or -independent.

p53-dependent induction of p21 transcription

As an effector gene of p53, p21 promoter contains two p53-responsive elements, whose sequences are highly conserved among mouse, rat and human (El-Deiry et al., 1995); one of these sites may suffice for p53 induction (Poluha et al., 1997; Wu and Schonthal, 1997). Several different conditions and factors are able to induce the transcription of p21 in a p53 dependent manner, but DNA-damaging agents, γ-irradiation in particular, often induce p21 p53-dependently (Macleod et al., 1995). Accordinly, spindle disrupter nocodazole, differentiation factor Neu, and ribonucleotide synthesis inhibitor PALA, to list just a few, induce p21 by activating or stabilizing p53 (reviewed by Gartel and Tyner, 1999). Quite controversial findings have been reported about the dependency of p53 on the activation of p21 by UV radiation. Some groups have found p53-independent p21 induction after UV-treatment (Loignon et al., 1997), while others have shown a lack of p21 expression in the absence of p53 (Gorospe et al., 1998).

Oncogenic Ras causes growth arrest in primary fibroblasts and leads to accumulation of p53, p21 and p16 (Serrano et al., 1997), but p21 induction by Ras may occur also in a p53 independent manner (Kivinen et al., 1999). Induction of p21 by Raf, a downstream effector of Ras, has been shown to occur both p53-dependently (Lloyd et al., 1997) and independently (Sewing et al., 1997; Woods et al., 1997).

p53-independent regulation of p21 expression

Differentiation promoting agents often induce p21 by p53-independent mechanisms by inducing the binding of different transcription factors to specific elements in p21 promoter. Human p21 promoter contains six Sp1-binding sites located within 120 bp upstream of transcription initiation site, and many p21 activating agents, for example tumor suppressor BRCA-1 (Somasundaram et al., 1997), utilize these binding sites.

Accordingly, p21 activation by TGF-ß is mediated by interaction of Sp1 with Smad proteins (Li et al., 1998; Moustakas and Kardassis, 1998). On the other hand, growth arrest and upregulation of p21 induced by EGF and IL-γ occur by STAT1 via several STAT-binding sites (Chin et al., 1996). In addition, specific binding sites for transcription factors E2Fs and AP2 exist in p21 promoter (Gartel and Tyner, 1999).

Although p21 regulation occurs mainly at the transcriptional level, posttranscriptional mechanisms are also possible. Both p21 mRNA (Gorospe et al., 1998) and protein (Timchenko et al., 1996) can be stabilized without an increase in its transcription. Posttranscriptional events have been suggested to possess a significant role in p21 regulation particularly after genotoxic stress (Butz et al., 1998).

CELL CYCLE REGULATORS IN MELANOMA

Malignant melanoma is one of the most common tumor types in western countries; only cancers of breast, prostate, colon, and lung occur more frequently than melanoma. Although epidemiological studies have strongly demonstrated that exposure to sunlight is the major environmental cause of melanoma, the exact molecular targets of UV radiation remain to be identified. The genetic loci 6q and 9p21 are often lost in cutaneous melanomas – the melanoma susceptibility locus at 9p21, including for example p16 gene locus, is absent in over half of malignant melanomas.

p53 changes in melanoma

Infrequent p53 mutations

In contrast to the majority of cancers, including other skin cancers, p53 mutations in melanoma are rare. p53 mutation frequency seems to correlate with the aggressiveness of the tumor, because p53 mutations are detected in less than 1% of primary melanomas (Hartmann et al., 1996), in 5% of metastatic melanomas (Florenes et al., 1994b; Akslen et al., 1998), and in approximately 20% of melanoma cell lines (Albino et al., 1994; Bae et al., 1996). This suggests that p53 mutation may not be the major underlaying cause in the development of melanoma, but instead have a role in the progression and invasiveness of this cancer type. Nevertheless, Li-Fraumeni patients with germline mutation of p53 are prone to several cancers, including melanomas (Platz et al., 1998) . In non-melanoma skin cancers, squamous and basal cell carcinomas, p53 mutations are frequent and appear early in the development of a tumor (Ziegler et al., 1994). In both tumor types, however, the observed p53 mutations are typically found at dipyrimidine sites and harbor the hallmarks of UV mutagenesis suggesting UV radiation as a

causative agent of transformed growth (Ziegler et al., 1993; Nakazava et al., 1994; Hartmann et al., 1996; Zerp et al., 1999). Moreover, in the case of melanoma metastases, p53 mutations were significantly more common in skin metastases than in those of internal organs, providing further evidence of UV-radiation induced mutagenesis of p53 (Zerp et al., 1999).

Overexpression of wild-type p53

Another characteristic feature of p53 in melanomas is the overexpression of wild-type p53. In the early days of p53 research high levels of normal p53 in melanoma samples were considered as a mutant protein, since p53 mutation screening was largely based on the detection of accumulated p53 protein. Direct DNA sequencing later revealed the wild-type nature of stabilized p53 and the low frequency of p53 mutations. Overexpression of wild-type p53 in melanomas approximates 60%, and has a tendency, like p53 mutations, to occur more commonly in metastatic than in primary melanoma cells (Lassam et al., 1993; McGregor et al., 1993; Florenes et al., 1995; Zerp et al., 1999). Interestingly, melanoma patients with overexpressed p53 had better prognosis than patients with normal p53 levels (Essner et al., 1998; Healy et al., 1998). Some studies, however, have failed to confirm this correlation (Korabiowska et al., 1997).

In individual melanoma cases overexpressed p53 has been found in the wrong cellular compartment (Weiss et al., 1995), but most often the localization is normal (Lassam et al., 1993). Melanomas are not unique in expressing high levels of normal p53, since it has been also detected in some chorioncarcinoma (Landers et al., 1994), bladder (Lianes et al., 1994) and sarcoma cells (Cordon-Cardo et al., 1994). In these tumors high levels of mdm2, but not other target genes, accompanied overexpressed p53, supposedly trying to suppress the effects of elevated levels of p53. Mdm2 overexpression in a variety of tumor cells has been shown to result from enhanced expression of mdm2 mRNA, in particular initiated from the internal p53-responsive promoter (Landers et al., 1997). Despite a normal sequence of stabilized p53, its functions, target gene induction and DNA binding ability, may be reduced (Bae et al., 1996).

p16 mutations

During the last years p16 cyclin kinase inhibitor has established its role as a tumor suppressor in familial melanoma as well as in many other cancers. The pedigrees of familial melanoma with several affected individuals in several generations are rare, but over 50% of these families carry a germline p16 mutation (Hussussian et al., 1994). Family members with hereditary susceptibility to melanoma, comprising 5-10% of all

melanoma patients, also have a positive family history with at least one affected relative. Germline p16 mutations are observed in about 20% of these patients (FitzGerald et al., 1996). Germline mutations are typically point mutations or small deletions that are located in the exon 1α thus affecting only p16 without interfering with $p19^{ARF}$ (Gruis et al., 1995; Holland et al., 1995; Walker et al., 1995).

Other

Two familial melanoma kindreds have been found to carry Cdk4 point mutations that make Cdk4 insensitive to the inhibitory actions of p16 (Zuo et al., 1996). Additionally, 92% of sporadic melanoma cell lines have been found to harbor inactivated p16 or Cdk4 proteins, stressing the importance of p16 inactivation in the development of melanoma (Castellano et al., 1997).

The role of p21 in melanomas is theoretically interesting for two reasons: p21 is located in the same area of chromosome 6 which often contains changes in melanoma, and p21 was originally cloned also as a melanoma differentiation associated gene (mda-6). Despite these connections, p21 mutations are practically never found in melanomas. In contrast to p53, p21 expression often decreases with tumor progression (Maelandsmo et al., 1996).

AIMS OF THE PRESENT STUDY

The DNA damage response of γ-irradiated cells has been thoroughly studied during the last few years, and the requirement of wild-type p53 in growth arrest upon ionizing radiation cannot be questioned. UV radiation, causing DNA lesions specific to UV light, results in a damage response that differs from that caused by ionizing irradiation both in kinetics and in the need for cell cycle regulators. Despite the established role of p53 as a gatekeeper in cell cycle checkpoints, its necessity in UV-induced growth arrest and its functions in specific cell cycle phases have not been elucidated.

The present study was undertaken to:

1) study the effects of UV radiation on growth arrest, p53 stabilization, and pRB phosphorylation
2) examine the dependency of UV-induced p53 accumulation and transactivation on cell cycle phase
3) explore the frequency of p53 mutations in melanoma cell lines and to determine the UV response in melanoma cell lines overexpressing wild-type p53
4) clarify the p53-independent mechanisms of p21 regulation after UV treatment

MATERIALS AND METHODS

Cell lines

The cell lines used are described in Table 1.

Table 1. p53 status and origin of cell lines used in the presented study.

Cell line	Description	p53 status	Source	Used in
NIH3T3	mouse fibroblast	wt	ATCC CRL 1658	I, II
CCl-137	human embryo lung fibroblast	wt	ATCC	I
WI-38 human embryo lung fibroblast		wt	ATCC CC175	I
T-24	bladder adenocarcinoma transformed by Val12-Gly ras	wt		I
SW480	human colon carcinoma	mut		I
3T3RB4.6	NIH3T3 transfected with human RB cDNA (driven by SV40 promoter)	wt	Pitkänen et al., 1993	I
3T3RB4.6p VU-0	NIH3T3 transfected with human RB and large T-antigen (driven by SV40 promoter)	wt	Pitkänen et al., 1993	I
A-375 human malignant melanoma		wt	ATCC CRL 1619	IV
Malme-3M	human metastatic melanoma	wt	ATCC HTB 64	IV
WM239	human malignant melanoma	wt	ATCC	IV
G361	human malignant melanoma	wt	ATCC CRL 1424	IV
RPMI-7951	human metastatic melanoma	mut (166S/stop)	ATCC HTB 66	IV
SK-MEL-2	human metastatic melanoma	mut (245G/S)	ATCC HTB 68	IV
SK-MEL-28	human malignant melanoma	mut (145L/R)	ATCC HTB 72	IV
p53+/+ MEF	mouse embryonic fibroblast	wt	Dr. L. Donehower	III
p53-/- MEF	p53-deficient MEF	wt	Dr. L. Donehower and Dr. K. Wiman	III
Melanocytes	primary human melanocytes	wt	Dr. O. Saksela	IV

Cell culture and cell synchronization

The cells were grown at 37°C in the presence of 5% CO_2 atmosphere and maintained in DMEM (Life Technologies Inc.) supplemented with either 10% newborn calf serum (NBCS; Gibco) (NIH3T3 and its derivatives) or in 10% fetal calf serum (FCS; Life Technologies Inc.) (CCL-137, WI-38, T-24, A-375, Malme-3M, G361, WM239, SK-MEL-28, p53-/- and +/+ MEFs). SW480 cells were cultured in RPMI 1640 medium in the presence of 10% FCS, and RPMI-7951 and SK-MEL-2 cells in MEM supplemented with 10% FSC and non-essential amino acids. Primary MEFs were used between passages 3 to 8, and human skin derived melanocytes below passage 10.

NIH3T3 cells were synchronized to G_0/G_1 by contact inhibition and serum starvation for 8 h in the presence of 0.2% NBCS and released to cycle by replating cells in culture medium containing 10% NBCS. Synchronization to G_1/S was performed by initial serum starvation as above, followed by addition of fresh medium in the presence of 0.25 mM hydroxyurea (HU) (Sigma) and incubation for 16 h. After removal of HU-block the cells entered synchoronously into S. HU alone had no effect on p53 levels nor p53 DNA-binding activity.

UV treatment of cells

For UV treatment, medium was removed and the cells were exposed to UVC (254 nm) at a dose of 10 - 200 J/m^2 with UV-Stratalinker 1800 (I, II) or 2400 (III, IV) (Stratagene). Fresh medium was added, also to unradiated control cells, and cells were incubated for the indicated periods of time before analysis.

Immunoblotting

For semiquantitative analysis of protein by immunoblotting the cells were washed with cold 25 mM Tris-HCl (pH 8.0), containing 150 mM NaCl (Tris buffered saline, TBS), and lysed with 25 mM Tris-HCl (pH 8.0), containing 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 4 mM NaF, 100 μM Na_3VO_4, 100 KIU/ml aprotinin, 1 mM PMSF, and 10 μg/ml leupeptin at 4°C for 25 min. Cell lysates were clarified by microcentrifugation, and protein concentrations were determined by Bradford analysis. Lysates (200 - 500 μg) were analysed by 7.5% or 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions followed by transfer of proteins to Immobilon-P^{TM} membranes (Millipore). The membranes were probed either with monoclonal antibody PMG3-245 (PharMingen) against pRB, monoclonal DO-1 (Santa Cruz Biotechnology) or monoclonal PAb240 (PharMingen) against human and mouse p53, respectively, monoclonal 6B6 (PharMingen) against human p21, or either

polyclonal M-19 (Santa Cruz Biotechnology) or polyclonal 13436E (PharMingen) against mouse p21. PAb240 recognizes only mutant p53 under non-denaturing conditions, but both mutant and wild-type p53 under denaturing conditions. DO-1 antibody recognizes both forms of human p53. The antibodies were detected with peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL; Amersham). When indicated, densitometric scans of the immunoblots were analysed using NIH Image 1.47 program. Even loading of samples to polyacrylamide gel was verified by staining parts of the gel with Coomassie Brilliant Blue and of membranes by Ponceau S.

Immunofluorescence and 5-BrdUrd incorporation

Cells were grown on glass coverslips, fixed with methanol (MeOH) and acetone (1:1) for 5 min and stained for 1 h with primary antibody PMG3-245 to detect pRB, PAb 246 (PharMingen) or DO-1 to detect mouse and human p53, respectively. Subsequently, the coverslips were incubated with rhodamine-conjugated rabbit anti-mouse antibody (Dako), and nuclei were counterstained with 2 μg/ml Hoechst 33258. The stainings were visualised with 100 x magnification under UV illumination with an Olympus BH-2 microscope.

DNA replication was determined by 5-bromo-2´-deoxyuridine (5-BrdUrd) incorporation. Cells grown on coverslips were incubated with 50 μM 5-BrdU (Sigma) for 1 or 2 h and fixed with ice cold MeOH for 5 min. After permeabilisation of cell membranes with 1.5 N HCl for 20 min, cells were stained with monoclonal 5-BrdUrd antibody (Amersham) and rhodamine-conjugated rabbit anti-mouse antibody (Dako), both for 1 h. Nuclei were stained with 2 μg/ml Hoechst 33258 for 2 min and after visualisation with a microscope, the proportion of 5-BrdUrd positive nuclei of all Hoechst dye-stained nuclei was determined.

Northern (RNA) blotting

Poly(A)$^+$ mRNA was isolated from cells by oligo(dT) cellulose, separated in 1% agarose gels containing formaldehyde and transferred to Hybond-N membrane (Amersham) in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). mRNA was detected by probing with p21 (El-Deiry et al., 1993), GADD45 (Fornace et al., 1988), or mdm2 cDNA inserts (kindly provided by Dr. B. Vogelstein, Dr. A. J. Fornace Jr., and Dr. A. Levine, respectively) labeled with α[^{32}P]dCTP by random priming. Quantitations of the autoradiograms were carried out with Fujifilm BAS-1500 image analyser and the MacBAS 2.1 program. Fold inductions were calculated by normalizing the mRNA levels to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and comparing the signals of the UV-treated and control cells.

Flow cytometry

For analysis of cell cycle phase distribution by flow cytometry the cells were trypsinized, centrifuged for 5 min at 1500 rpm, washed with phosphate-buffered saline (PBS), fixed with ice cold MeOH and stored at -20°C. Subsequently, thawed cells were centrifuged, washed with PBS, resuspended in 0.5 ml of PBS containing 50 μg/ml RNase A (Sigma), and incubated for 30 min at 37°C. DNA was stained with 50 μg/ml propidium iodide (Sigma) overnight, and flow cytometry analysis was performed by FACScan (Becton-Dickinson). The data of cell cycle distribution was analyzed using the CellFIT Cell Cycle Analysis program.

Assays for apoptosis

UV-treatment induced morphological changes of nuclei, apoptotic nuclear condensation and fragmentation, were estimated from cells fixed on glass coverslips and stained with DNA dye Hoechst 33258 (Sigma) at a concentration of 2 μg/ml for 2 min, and the extent of apoptotic changes was visualised with an Olympus BH-2 microscope under UV illumination. In addition, A0-cell population, representing apoptotic cells with less than 2N DNA content, was determined by flow cytometry analysis.

Electrophoretic gel mobility shift assay

Nuclear extracts were prepared as described previously (Andrews et al., 1991). Oligonucleotides representing the consensus p53 binding sites either in the $p21^{CIP-1/WAF-1}$ gene promoter (El-Deiry et al., 1992), 5´-AATTCTCGAGGAACA-TGTCCCAACATGTTGCTCGG-3´, or mutated binding site 5´-GAATTCTCGAGG-AAAATTTCCCAAAATTTTGCTCGAG-3´ were synthesized, annealed into double-stranded form and labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs). Oligonucleotide probes for wild-type (ATTCGGTCCCGCCTCCTTGAG-AGC) or mutant (ATTCGGTCCCGGAATCCTTGAGAGC) Sp1-binding sites were prepared similarly. Binding reactions contained 10 μg of nuclear extract, 10 μl of 2x binding buffer [40 mM Hepes-KOH pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 4 mM MgCl, 1 mM dithiothreitol, 0.05% NP-40, 4 mM spermidine (Sigma), and 100 ng poly(deoxyinosinic-deoxycytidylic acid) (Pharmacia)], and, when indicated, 500 ng of specific antibody in a final volume of 20 μl. Binding reactions were incubated at room temperature for 20 min, 0.2 ng of labeled oligonucleotide probe was added and the incubation was continued for an additional 20 min at room temperature. Reaction products were separated on a 4% nondenaturing polyacrylamide gel with

0.25X Tris-borate-EDTA buffer supplemented with 5% glycerol at 4˚C. After drying, the gel was exposed to X-ray film.

Cell transfections and chloramphenicol acetyltransferase (CAT) assay

NIH 3T3 cells were co-transfected with PG13-CAT or MG15-CAT constructs (kindly provided by Dr. B. Vogelstein) (Kern et al., 1992) and with pcDneo neomycin resistance gene (Chen et al., 1987) by the calcium phosphate precipitation method and grown in the presence of 0.6 mg/ml G418 (Gibco) for two weeks. Stable cell colonies from both transfections were trypsinized and pooled. For measurement of CAT-activity the cells were pelleted, lysed and equal concentrations of protein were incubated with acetyl coenzyme A (Pharmacia) and 0.4 μCi of [^{14}C]chloramphenicol (Amersham). The acetylated chloramphenicol was separated by thin-layer chromatography (Gorman et al., 1982), and signals of the acetylated forms of [^{14}C]chloramphenicol were quantitated with a Fujifilm BAS-1500 image analyzer.

p53 sequencing

Reverse transcription-polymerase chain reaction (RT-PCR) was used to generate cDNA, and all DNAs representing exons 1-11 of p53 gene were sequenced. For RT-PCR, two sets of primers generating a terminal, 774-bp fragment (forward primer, CTGCTGGGCTCCGGGGACACTTTG; reverse primer AGGCGGCTCATAGGG-CACCACCAC) and a COOH-terminal 890 bp fragment (forward primer, TACTCC-CCTGCCCTCAACAAGATG; reverse primer TTCAAAGACCCAAAACCCAAA-ATG) were used. cDNA was sequenced from both strands using automated DNA sequencing, and the sequences were compared against p53 cDNAs in the databases.

p21 promoter analyses

NIH3T3 and p53-/- MEFs were transiently transfected by the calcium phosphate precipitation method with p21 promoter deletion constructs, which were a kind gift from Dr. X.-F. Wang (Datto et al., 1995). Additionally, p21 promoter constructs containing two or three mutated Sp1-binding sites (mut 2+4, TATCTAGAAC TGAGGCGGGC ATCTAGACAT [-83 - -54]; and mut 2+3+4, TATCTAGAAC CTCTAGAAAT ATCTAGACAT [-83- -54]) were generated using one mutated Sp1-binding site containing construct (mut 2, mut 3, or mut 4) as the template in two successive PCR reactions with appropriate primers. The identities of PCR products were verified by sequencing and then cloned into 93-S vector (Kivinen et al., 1999). The cells were treated with UV radiation 24 h after transfection and after additional 20 h incubation luciferase activity was determined by Dual-Luciferase reporter assay

system (Promega). As an internal control of transfection efficiencies pRL-TK expression plasmid coding for *Renilla* luciferase was included in each transfection.

RESULTS AND DISCUSSION

Transient growth arrest induced by UV radiation (I, II, III, IV)

The effects of UV radiation on the growing ability and induction of apoptosis was studied in diverse cellular contexts, in both human and mouse cell lines with normal, defective or no p53. Regardless of the different genetic backgrounds, UVC radiation of 254 nm appeared to be an efficient inducer of transitory growth arrest in every cell line studied.

G1- and S-phase arrests after UV

The effect of 50 J/m ^{2} UVC radiation on DNA replication was first examined in exponentially growing mouse NIH3T3 fibroblasts. As measured by 5-bromo-2´-deoxyuridine (5-BrdUrd) nucleotide analog incorporation, cells underwent a transient growth arrest between 6 h and 12 h after irradiation followed by a gradual increase in replication from 12 h onwards (I). However, flow cytometry analyses from 6 h and 12 h timepoints indicated that despite inhibition of DNA replication cell cycle phase distribution was identical in radiated and unradiated cell populations, demonstrating that instead of gathering into G1 the radiated cells arrested in the phase they were at the time of radiation (unpublished observation).

To examine the kinetics of UV-induced growth arrest – as well as activation of tumor suppressors pRB and p53 – in specific cell cycle phases, NIH3T3 cells were synchronized by contact inhibition and serum starvation to the G0/G1 phase, replated, and stimulated with normal medium thus allowing cells to progress from G1 to S. Flow cytometry analyses revealed that if the cells were radiated in G1 or the G1/S border, they arrested in that phase for 10 to 12 h before entering back into the cell cycle. However, if the cells were exposed to UV while in S or early G2/M, the arrest period was considerably longer and cells failed to recover from the insult during follow-up unlike G1-phase cells (I).

An even more detailed study of growth arrest kinetics was carried out with NIH3T3 cells that were synchronized to G0/G1 and replated in the presence of hydroxyurea (HU) to inhibit the progression to S. Synchronized cells were treated with 50 J/m^2 of UV either at this artificial G1/S border or, after release from HU-block, in different S-phase stages (II). Information combined from 5-BrdUrd incorporation analyses and flow cytometry profiles indicated that during the 6 h incubation time after radiation the cells were able to minimally progress in the S phase. Accordingly, DNA replication did not cease instantly but decreased gradually becoming negligible during the last incubation hour. HU is an inhibitor of ribonucleotide reductase depleting the nucleotide

pool available and thus inhibiting the DNA replication. When it was added to the medium, cell cycle progression and DNA replication of all UV-radiated cells were inhibited (II).

Diverse DNA damaging conditions including UV radiation lead to growth arrest of cells in order to provide sufficient time for repair of the damaged DNA and maintenance of genomic stability. As found by us and others, UV-induced inhibition of growth is rapid, beginning almost immediately after the insult. Pyrimidine dimers and photoproducts caused by UV radiation impair the replication by distorting the DNA conformation Taylor et al., 1988; Taylor et al., 1990) and, at the same time, activate regulatory pathways involving damage recognition and checkpoint control. Inhibition of DNA replication upon high dose UV comprises inhibition of replicon initiation and reduction in the rate of chain elongation, the former being inhibited by 50% within the first hour after exposure to UV (reviewed by Frieberg, 1995).

Cell cycle phase synchronization is absolutely essential in order to separate the cell cycle responses of exponentially growing cells representing all cycle phases. As suggested by results obtained from exponentially growing cells (I), we demonstrated that after UV exposure the cell cycle can be halted in either G1 or S phases depending on the original stage of cells, and after insult the cells progressed in the cycle only little if at all (I, II). Moreover, S phase arrest appeared longer than G1 arrest as if more time was required for repair of S-phase lesions. Contrary to what might be expected, the apoptosis rate was not significantly higher in S-arrested cells (unpublished observation).

It has been demonstrated years ago that mutations due to UV radiation are considerably fewer if cells are prevented from entering the S phase, for example by HU (Stone-Wolff and Rossman, 1982). Due to active replication in S, repair of UV lesions may be less efficient in this phase leaving more mutations unrepaired. In addition to impaired repair, mutations are also fixed to the genome during subsequent DNA replication. Studies with synchronized normal fibroblasts have demostrated that the longer the period between UV and entry into S, the less the mutations accumulate (Konze-Thomas et al., 1982). The disappearance of premutagenic lesions before S is probably based on nucleotide excision repair, since XP cells deficient in NER display the same rate of mutations regardless of the length of G1 phase preceding S. Consistent with our findings, final cell survival is not, however, affected by the time point of UV radiation in either cell lines, suggesting that other repair mechanisms may in part overtake the functions of NER (Konze-Thomas et al., 1982). When regarding mutagenesis, a wave length of 254 nm has proven to be most efficient for cells in the S phase or its vicinity (Kaufmann, 1989).

Growth arrest and apoptosis in the absence of functional p53

The dependency of UV-induced growth arrrest on p53 functions was examined in multiple cell lines with different p53 status. Besides normal human and mouse fibroblasts, growth arrest upon UV treatment was observed also in cell lines with mutant p53 (SW480) or with p53 inactivated by SV40 large T antigen (RB4.6pVU-0) with at least 60% inhibition of DNA replication (I).

p53 coding exons of seven melanoma cell lines were sequenced, and three cell lines were found to carry mutant p53, one of them expressing a truncated form of p53 protein (IV). As determined by 5-BrdUrd incorporation, all melanoma cell lines underwent a growth arrest after 50 J/m^2 of UVC with at least 60% inhibition of DNA replication. Consistent with findings in NIH3T3 cells, the arrest responses of melanoma cell lines peaked at 6 to 16 h after irradiation, after which two alternative responses were found: three cell lines (A-375, WM239, and SK-MEL-2) remained arrrested for 24 h of incubation, while four lines (G361, Malme-3M, SK-MEL-28, and RPMI-7951) restored the cellular growth, RPMI-7951 with truncated p53 protein did so only partially. Melanoma cell lines permanently arrested and incapable of entering cell cycle also harbored markers of apoptosis. Melanocytes, used as control cells and an example of untransformed primary skin cells, took a course of their own; although lacking detectable DNA replication at 24 h after radiation they were able to recover at 30 h postirradiation and displayed only a few apoptotic cells. Most interestingly, neither growth arrest kinetics nor apoptosis pattern were dependent on p53 status. It should be remembered, however, that the UV response of wild-type p53 melanoma cell lines cannot be necessarily regarded as normal, since their p53 pathway may be defective due to accumulated wild-type p53. Nevertheless, UVC was shown to result in growth arrest in melanoma cell lines irrespective of functional p53 (IV).

Inspired by our previous findings we explored the growth arrest of cells totally lacking p53. Unsynchronized p53-/- MEFs from p53 knockout mice and p53 positive normal MEFs were exposed to 25 and 50 J/m^2 of UVC. Both cell lines were arrested with similar kinetics, maximal inhibition of DNA replication occurring 6 h after insult correlating with that seen in NIH3T3 cells. However, p53-/- cells were permanently arrested, and a considerable fraction of cells (approximately 15% with 25 J/m^2 and 35% with 50 J/m^2) underwent apoptosis, whereas p53+/+ cells reentered the cell cycle by 24 h after UV radiation with less than 3% of cells exibiting apoptotic features (III). These findings involving both melanoma cells and p53-/- MEFs demonstrated that functional p53 is not required for UVC-induced growth arrest.

In contrast to UV radiation, functional p53 is required for successful growth arrest after γ-irradiation (Kastan et al., 1991; Kastan et al., 1992; Kuerbitz et al., 1992; Dulic et al., 1994). However, several lines of evidence have demonstrated that it is not

necessarily needed for apoptosis induced by γ-radiation or by other signals (Clarke et al., 1993; Kelley et al., 1994). Although p53 has been suggested to be essential for growth arrest after UV radiation as well, direct evidence of its requirement has been missing. On the contrary, UVC has been shown to induce G1 growth arrest in p53 deficient Li-Fraumeni cells (Loignon et al., 1997) being in accordance with our results for p53-/- MEFs (III). Recently it was reported that HaCaT cells lacking p53 ceased to proliferate when exposed to UVC during G0/G1 but not during S or G2/M suggesting that in the absence of p53 cells failed to undergo G2/M arrest but maintained the capacity for G1 arrest (Merryman, 1999). Our findings do not support the role of p53 in immediate, UV-triggered growth arrest, since growth inhibition was intact also in cells with defective or absent p53 (I, III, IV). However, after immediate inhibition of DNA replication, p53 -/- and +/+ cells followed different pathways. In contrast to normal MEFs, radiated p53 -/- MEFs were unable to re-enter cell cycle and were prone to apoptosis (III). Since apoptosis in -/- MEFs was more prominent with higher UV doses, programmed cell death can be speculated to occur due to defective DNA repair and accumulation of unrepaired DNA lesions in cells lacking p53. On the contrary, however, DeFrank et al. (1996) found no difference in survival between p53 null and p53+/+ cells. Similarly, apoptosis in UV-exposed melanoma cell lines (IV) and hepatocytes (Bellamy et al., 1997) displayed no correlation with p53 function.

Although p53 failed to be vital for successful growth arrest after UV radiation, some other cell cycle regulators – though closely related to the p53 pathway – were found to correlate with growth inhibition temporally; most of these are discussed in separate sections and are only listed here. One of our first observations was that G1 growth arrest was associated with pRB dephosphorylation and not with p53 accumulation (I). In melanoma studies, GADD45 mRNA induction was simultaneous with growth arrest and occurred in all melanoma cell lines regardless of p53 status. Moreover, high levels of GADD45 mRNA was associated with prominent apoptosis (IV). Another target gene of p53, p21, was induced in synchronized mouse fibroblasts during G1 growth arrest (II), and p21 mRNA and protein were overexpressed in UV-radiated p53-/- MEFs (III) suggesting a role for p21 in the transmission of growth arrest. However, in melanoma cell lines growth arrest failed to parallel p21 induction (IV). Depending on cell type and genetic backround, several pathways are undoubtedly involved in UV-induced growth arrest.

Function of pRB in G1-phase growth arrest (I)

Since p53 was known to accumulate in response to γ- and UV radiation (Lu and Lane, 1993; Zhan et al., 1993), we examined whether pRB, an other tumor suppressor

and cell cycle regulator, has any role in immediate cell response to UVC. As expected, p53 protein accumulated within 6 h after radiation with 50 to 100 J/m^2 in exponentially growing mouse (NIH3T3) and human fibroblasts (CCL-137, WI-38) (I). At the same time, pRB in radiated cells was found exclusively in the dephosphorylated, active form while unradiated control cells contained also hyperphosphorylated forms of pRB. Besides fibroblasts, dose-dependent pRB hypophosporylation upon UV insult was detected in all cell lines studied, including cells with mutant or inactive p53 (I). Hypophosphorylation of pRB correlated temporally with growth arrest kinetics in unsynchronized, UV-treated NIH3T3 cells. In addition, synchronized NIH3T3 cells radiated in G1 exhibited only hypophosphorylated pRb, and, to our initial surprise, no p53 accumulation occurred during the subsequent G1 proliferative arrest. Consistent with general growth arrest in unsynchronized cells, specific G1 arrest and pRB dephosphorylation attenuated with identical kinetics (I).

pRB hypophosphorylation is frequently detected in cells growth arrested by diverse genotoxic or growth restricting agents, but our study was one of the first to report its involvement in UV-induced growth arrest (I; Medrano et al., 1995). All DNA damage types do not lead to pRB hypophosphorylation; γ-irradiation failed to dephosphorylate pRB in p53-/- fibroblasts suggesting that the nature of the damage influences pRB phosphorylation changes (Dulic et al., 1994). This approach with synchronized cells allowed us to study pure G1 or S phase arrest and the functions of cell cycle regulators pRB and p53 in those phases.

In theory, pRB dephosphorylation in radiated cells could have been a secondary change caused by p53-mediated G1 growth arrest. However, two findings in our studies favored the p53-independent role of pRB in growth inhibition: first, pRB hypophosphorylation – and growth arrest – occurred also in cell lines harboring mutant or inactivated p53, and secondly, the kinetics of growth arrest in unsynchronized NIH3T3 cells correlated with that of pRB hypophosphorylation (I). Rephosphorylation of pRB 12 h after UV treatment, when DNA replication was still dampened, preceded, not followed, the entry of cells to the cycle. Moreover, in synchronized UV-treated NIH3T3 cells G1-phase growth arrest endured as long as pRB remained hypophosphorylated, but p53 accumulation was delayed until the cells recovered from G1 arrest and entered S phase (I).

These results thus not only demonstrate the role of pRB in UV-induced growth arrest in G1 but also question the participation of p53 in that particular phase (I). Our observation of pRB as a central mediator of the G1/S checkpoint proved to be in accordance with recent findings that exposure of RB-/- MEFs to various DNA damaging stimuli including UV, ionizing radiation, and chemotherapeutic agents resulted in defective cell cycle arrests (Harrington et al., 1998). It is particularly

interesting that also γ-irradiation, which has been traditionally strongly coupled to p53 function, failed to cause G1/S arrest in the absence of pRB despite increased levels of p53 and p21 (Harrington et al., 1998). On the other hand, polyamine analog treatment has been shown to raise the level of hypophosphorylated pRB, correlating temporally with G1 growth arrest as in our studies, but that the rise was preceded by p53 accumulation (Kramer et al., 1999). Thus, the nature of DNA damage caused by numerous growth suppressive agents is one of the most important determinants when cell cycle responses are interpreted.

NIH3T3 cells synchronized to and radiated in S phase remained arrested in S, and contained both hypo- and hyperphosphorylated pRB, whereas pRB in S-phase control cells consisted solely of hyperphosphorylated forms (I). Theoretically, it seems unlikely that pRB could influence S-phase arrest in S-phase cells, because phosphorylation events inactivating pRB have already taken place in late G1 or G1/S transition during the previous cycle. However, the existence of both phosphorylation forms of pRB in radiated S-phase cells as well as existence of only hypophosphorylated pRB in exponentially growing cells including cells in S (I), raises the question of UV-induced dephosphorylation of pRB during the S phase. Indeed, it was recently found that underphosphorylated pRB was present also in cells growth arrested by sodium butyrate in the S or G2/M phases (Yen and Sturgill, 1998), and in all cell cycle phases after hypoxia treatment (Danielsen et al., 1998), suggesting that the growth controlling capability of RB may not be limited to the G1 phase alone.

Dependency of p53 accumulation on cell cycle phase (I, II)

p53 accumulation in cell lines with wild-type p53

In response to a UVC of 50 J/m^2, accumulation of p53 in exponentially growing NIH3T3 and CCL-137 fibroblasts was observed by immunoblotting and immunofluoresence staining between 4 and 30 h after radiation, after which elevated levels of p53 gradually decreased (I; data not shown). Similarly, p53 accumulation occurred within 6 h after UV treatment in normal MEFs (III) and in melanoma cell lines expressing wild-type p53 (IV). On the contrary, cells harboring mutant (IV) or inactivated p53 (I) already expressing high levels of stabilized p53 failed to further accumulate it in response to UV. In most experiments cells were exposed to a UVC of 50 J/m^2, representing a sublethal dose with less than 5% of NIH3T3 cells undergoing apoptosis after 36 h incubation (unpublished data). Doses as high as 200 J/m^2 seriously damaged cells resulting in enhanced apoptosis and lower accumulation of p53 (I).

Accumulation of p53 upon UV radiation was first detected by Maltzman and Czyzyk as early as 1984 (Maltzman and Czyzyk, 1984). The kinetics of p53 accumulation in

NIH3T3 was in agreement with findings of others (Lu and Lane, 1993; Zhan et al., 1993), and differed from that seen after ionizing radiation. γ-irradiation causing direct double-strand breaks is a more rapid and more efficient inducer of p53 accumulation than UV radiation (Lu and Lane, 1993). In general, agents leading to DNA strand breaks, such as topoisomerase I inhibitors, γ-irradiation, drugs intercalating to DNA, and DNA nucleases are most potent inducers of p53 (Nelson and Kastan, 1994) . The C-terminal domain of p53 is known to bind to single-stranded DNA ends (Bakalkin et al., 1994) and DNA lesions (Lee et al., 1995) leading to an activation of transcriptional activity of p53. Since UV radiation causes DNA strand breaks only during repair processes, it is understandable that p53 response to UV radiation is kinetically slower but longer lasting than to ionizing radiation. Compared to ionizing radiation, a more extended accumulation of p53 after UV may correlate with the longer persistence of DNA lesions and ongoing repair processes and may hence be dependent on UV dose.

p53 accumulation requires replication of damaged DNA

Although G1-arrested cells lacked p53 stabilization, this was, however, detected in exponentially growing fibroblasts (I), indicating that cells in cell cycle phases other than G1 were responsible for elevated p53 levels. Indeed, in synchronized NIH3T3 cells p53 stabilization after UV radiation of 50 J/m^2 was detected in two circumstances, both involving the S phase. While absent during G1 arrest, p53 accumulation was detected when G1-arrested cells recovered from UV insult and entered the S phase (I). Alternatively, if cells were UV treated while in the G1/S-phase border or S, p53 stabilized without delay and correlated with S-phase arrest (I, II). These observations are very suggestive that not the stage in the cell cycle when the insult is encountered but the replication of damaged DNA could be a prerequisite for p53 stabilization. Furthermore, regarding p53 accumulation, p53 seemed to play an essential part rather in S and G2/M arrest than in G1 arrest.

To examine the temporal correlation between p53 stabilization and DNA replication in more detail, a double block system was used to synchronize NIH3T3 cells either to G1, the G1/S border, early- or mid-S phase (II). The 5-BrdUrd incorporation percentage of cells UV treated in G1 or the G1/S border and incubated for 6 h correlated with accumulation of p53 analyzed by both immunofluoresence staining and western blotting; p53 level was low or undetectable when less than 5% of radiated cells replicated DNA, and p53 level was high (50% of cells p53 immunopositive) when 70% of cells were replicating at the time of UV insult (II). To further demonstrate that p53 accumulation was dependent on DNA replication rather than on the S phase itself, HU was added to the medium after exposure to UV. HU treatment was able to abolish the p53 accumulation in S-phase cells, but interestingly, it failed to decrease p53 levels in

cells radiated at the G1/S border; on the contrary, even higher amount of p53 was detected in the presence of HU in these cells (II).

Studies examining the cell cycle phase-dependent regulation of p53 accumulation by UV treatment had not been carried out before. We demonstrated that p53 accumulation after UV was cell cycle phase-dependent occurring only in cells radiated in S or G1/S phases or entering S after G1 arrest, and that accumulation correlated with DNA replication (I, II). Hypoxia, an other effective inducer of p53, has been later shown to induce p53 accumulation more efficiently in the S than in G1 or G2/M phases (Danielsen et al., 1998). The absence of stabilization of p53 during G1-phase growth arrest was unexpected (I), because p53 has been considered to be a G1 checkpoint controller. Assuming that p53 accumulation is dependent on recognition of DNA strand breaks, UV-type DNA lesions in G1-arrested cells may not be properly recognized by p53. In such a situation G1 arrest is perhaps maintained by other cell cycle regulators, such as pRB.

Replication of damaged DNA either in cells UV radiated in S or entering it after G1 arrest seemed to be a precondition for p53 accumulation. The replication dependency of p53 accumulation by UV is consistent with previous findings (Nelson and Kastan, 1994), but detailed analysis of p53 accumulation in different cell cycle stages had not been reported previously. Besides UV radiation, topoisomerase I inhibitor camptothecin requires DNA replication for p53 stabilization (Nelson and Kastan, 1994) suggesting that if a DNA damaging agent does not directly cause DNA strand breaks, the lesions should be at least exposed during replication. Damage signals evoked by UV in G1 may be recognized by p53 only in replicating DNA when it is processed to single-stranded DNA.

p53 accumulation in cells irradiated at the G1/S border was different from that seen in G1- or S-phase cell populations. Irradiated G1/S phase cells displayed accumulated p53, but in contrast to S-phase cells, p53 levels remained high in the presence of HU. However, G1/S time point fails to represent a normal cell cycle stage, because G1 cells that would otherwise continue to S are forced by HU to stop at the threshold of S. At the time of radiation these cells have opened replication forks, but a lack of nucleotides inhibits DNA replication but not NER. At this stage p53 stabilization independent of replication may be based on interaction of p53 with other cellular proteins. In fact, it has been later shown that p53 accumulation in G1/S, as well as in other phases, requires protein synthesis, since treatment with cycloheximide, an inhibitor of protein synthesis, totally prevented p53 accumulation in the presence and absence of HU (Pitkänen et al., 1998). It should be pointed out that up to the present, these findings apply only to these UVC-radiated mouse fibroblasts.

Dissociation between transactivation and accumulation of p53 (II)

Regulation of p53 transactivation in UV-radiated cells in different cell cycle phases turned out to be different from that of p53 accumulation. As in earlier studies, NIH3T3 mouse fibroblasts were exposed to 50J/m^2 of UVC in diverse stages of the cell cycle, and transactivation determinants, such as DNA binding activity, transcriptional activity as determined by chloramphenicol acetyltransferase (CAT) assay, and induction of target gene mRNAs, were compared to growth arrest kinetics and p53 accumulation. First, however, protein levels of p21 CKI were determined in cells irradiated in the G0, G1, G1/S, and S phases; G1/S represented the time point where G1 cells were inhibited by HU from entering S. In contrast to p53 protein which lacked accumulation in the G0 and G1 phases, p21 protein was induced in these and in all other stages (II). After UV radiation HU treatment abolished p21 protein induction in S but not at G1/S, a situation identical to that of p53 accumulation (II).

Since p21 protein was induced both in the presence and absence of accumulated p53, mRNA levels of two p53 target genes, p21 and GADD45, were measured in different cell cycle phases with and without HU. p21 and GADD45 mRNA levels were induced by UV radiation in all cell cycle phases. Compared to non-irradiated cells maximum induction of p21 mRNA ranged from 8-fold in G1 to 3-fold in S, GADD45 induction being 3-fold and 2-fold, respectively. Inhibition of replication by HU abolished mRNA inductions of both p21 and GADD45, including G1/S-phase cells (II).

The capability of p53 to specifically bind to the p53-responsive element and induce transcription was explored using p53-responsive reporter constructs. NIH3T3 cells were stably transfected with the reporter plasmid construct PG13-CAT, and the effect of UV on transcriptional activity of p53 was measured as an increase in CAT activity. It reached on average a 6-fold increase in G0/G1 UV-treated cells and 3-fold increase in G1/S- and S-phase cells without any inhibitory effect by HU. Transfection with MG15-CAT, containing mutant p53-binding sites, resulted in negligible CAT activities (II).

Finally, the sequence specific DNA-binding activity of p53 was assayed by EMSA. UV-activated p53 protein binds to a radiolabeled oligonucleotide containing consensus p53-binding site from p21 promoter, and the number of complexes containing active p53 was estimated after separation in polyacrylamide gel. EMSA results were consistent with the findings in CAT-assay: specific DNA binding activity of p53 was increased by UV in all cell cycle phases despite the presence of HU (II).

These results indicate that p53 transactivation is dissociated from p53 accumulation, since transcriptional activition was observed by all three methods in the G0 and G1 phases in the absence of stabilized p53. Moreover, transactivation appeared to be clearly independent of the cell cycle phase, and, according to CAT-assay and EMSA,

independent of DNA replication, because HU treatment in the S phase had no effect on DNA binding activity of p53. However, p21 and GADD45 mRNA as well as p21 protein were not induced in S in the presence of HU suggesting a role for accumulated p53 in these processes. On the other hand, neither the absence of replication nor p53 accumulation impaired the increase in p21 and GADD45 mRNA levels in G1 phase (II).

The three ways employed to analyze the transcativation function of p53 have their limitations. Mobility shift assay displays only the amount of p53 protein bound to the p53 consensus binding site. Although being highly specific for p53, it fails to measure activation of transcription. Increase in CAT activity, on the other hand, has been preceded by DNA binding and the activation of transcription by p53. Inductions of target gene mRNA and protein are close to physiological signals, but they are subject to diverse posttranscriptional and posttranslational modifications independent of p53. Thus, EMSA and CAT-assay are highly specific for p53 but may be too simplistic, whereas evaluation of target gene induction does not require artificial interventions such as transfections, but may in turn be a too complicated method to explore p53 function alone. Nevertheless, information combined from these assays provide indications of the regulation of transcriptional activity of p53.

Traditionally, p53 accumulation has been considered to be a consequence of p53 activation due to inhibition of protein degradation, and accumulation and transactivation have been regarded as simultaneous and interdependent events. However, coinciding with our report, discordance has been found between transcriptional activity and accumulation of p53 at low UV doses; 10 J/m^2, but not higher doses, resulted in enhanced transcriptional activity without an increase in p53 protein (Lu et al., 1996). Although p53 accumulation after UV radiation seems to require replication of damaged DNA, other signals must be involved for its transcriptional activation. Regulation of p53 accumulation may depend on the ability of mdm2 to bind and target p53 for destruction, whereas other events, particularly protein modification by phosphorylation, may regulate the transcriptional activity of p53. In fact, mice lacking DNA-PK, a kinase phosphorylating Ser15 and 37, showed normal p53 accumulation but no activation of p53 by ionizing radiation (Woo et al., 1998). Discordance between accumulation and transactivation suggests that p53 accumulation is involved in recognition of unrepaired DNA lesions in the S phase. Supporting this hypothesis, NER-deficient cells accumulated p53 at much lower UV doses and exhibited a more prolonged response compared to normal cells or cells with restored NER activity, suggesting that p53 accumulation does not correlate with the NER process itself but with the presence of unrepaired cyclobutane dimers (Dumaz et al., 1997; Dumaz et al., 1998). The finding that p53 is transcriptionally active – although not accumulated – throughout the cycle,

support its role as a checkpoint controller in all phases of the cycle including G1. Whether p53 accumulation alone has a function independent of p53 transactivation still lacks direct evidence and remains to be clarified.

p53-independent induction of p21 upon UV treatment (II, III, IV)

Regulation of p21 by UV radiation in synchronized NIH3T3 cells showed many interesting aspects. Contrary to p53 accumulation, p21 protein was induced in all cell cycle phases (II) suggesting its participation in both G1- and S-phase arrests via its Cdk-inhibiting or PCNA-binding functions (Chen et al., 1995; Luo et al., 1995). Although the DNA-binding activity of p53 was not dependent on DNA replication, p21 protein, its mRNA and GADD45 mRNA were upregulated in the S phase only in replicating cells (II). It seems that target gene induction requires more than binding of activated p53 to its responsive element and that these other events, whether posttranscriptional modifications or stabilization of p53, are prevented by HU. Alternatively, UV radiation can induce p21 and GADD45 independently of p53, but p53 may still augment this effect. p53-independent transcriptional regulation of p21 gene is frequently observed, although most commonly in conditions other than genotoxic stress (Chin et al., 1996; Somasundaram et al., 1997; Li et al., 1998; Moustakas and Kardassis, 1998). Additionally, cells radiated at the G1/S border showed peculiar features. HU treatment prevented p21 mRNA induction but it had no effect on p21 protein induction (II). This dissociation of p21 protein and mRNA induction has been also demonstrated in breast epithelial cells after ionizing radiation (Gudas et al., 1995) and in growth arrested fibroblasts after serum stimulation (Macleod et al., 1995), where high p21 protein levels have been detected without an increase in p21 mRNA.

The observed discordance between the induction of p21 and activation or accumulation of p53 (II) were suggestive of p53-independent regulation of p21 after UVC radiation. Similarly, a melanoma cell line carrying mutant p53 was able to induce p21 mRNA in response to UV (IV). Since mutant p53 proteins may have retained some functions of wild-type p53, p21 regulation was studied in the total absence of p53 in p53-/- MEFs (III).

As described in the context of growth arrest, UVC responses of low passage p53-/- and p53+/+ MEFs were compared with respect to growth arrest and p21 induction. UVC doses of 10 to 50 J/m2, that are known to be sufficient for induction of p53 transcriptional activity, resulted in rapid increase in p21 protein in both cell lines. p53 +/+ cells, expressing higher p21 basal level than -/- cells, retained high p21 protein induction with 50 J/m^2, whereas the same dose in -/- MEFs yielded an attenuated p21

induction. The p53 accumulation pattern in p53+/+ MEFs followed the course observed in NIH3T3 cells and paralleled p21 induction (I, III).

Because elevation of p21 protein levels can be caused by transcriptional activation or by posttranslational modifications (Timchenko et al., 1996), mRNA levels in both cell types were evaluated. p21 mRNA was induced during the first 6 h after UV also in the absence of p53. When compared to unirradiated cells an over 5-fold induction was obtained within 16 h in both cell lines (III).

The transcriptional activation of p21 by UV radiation was further examined by studying the activation of p21 promoter. p53-/- cells were transiently transfected with p21 promoter deletion constructs containing luciferase gene as a reporter. Luciferase activity was measured from radiated (25 J/m^2) and control cells 24 h after UV. Transfection with full length p21 2.4 kb promoter construct, as well as with three shorter constructs lacking either one or two p53 binding sites, resulted on the average in 6-fold induction of luciferase activity by UVC confirming the p53-independent transcriptional activation of p21 by UV radiation (III).

As p53-/- cells are incapable of activating p53 binding sites we examined whether wild-type p53 in NIH3T3 cells was able to further increase the p21 promoter activity. UVC treatment led to 4-fold increase in reporter gene activity with both full-length p21 promoter and with a construct devoid of major p53 consensus binding site. Moreover, a construct with 400 bp 5´ deletion resulted in even greater induction of over 6-fold suggesting a negative regulatory element in region between -2.25 kb and -2.0 kb relative to the trancription initiation site. Deletion constructs containing DNA 500 to 110 bp upstream of the transcription initiation site showed a somewhat decreased UV inducibility, while only the shortest construct containing the last 61 bp totally abolished the UV-induced luciferase activity (III).

UV-induced transcriptional activation of p21 appeared to be most strongly regulated by a p21 promoter region between -110 bp and -61 bp relative to the transcription initiation site. Since the -110 bp area comprises five Sp1-binding sites, Sp1-binding site mutation constructs containing 93 bp were developed (Kivinen et al., 1999) and used to explore the effect of these sites on p21 induction. Of five Sp1-binding sites mutation of sites 2 and 4 decreased the UV inducibility, while the combined mutations of the two sites resulted in a complete loss of UV response. EMSA studies confirmed the sequence specific binding of Sp3 transcription factor to Sp1-binding site. In conclusion, UV-induced transcriptional activity of p21 was linked to two Sp1-binding sites in the vicinity of transcription initiation site, while a weaker regulation area was present between -1300 and -500 bp relative to transcription start. (III)

Hence, these studies indicated that UVC radiation induces p21 protein and mRNA expression and transcriptionally activates p21 promoter constructs also in the absence of

p53. While examples of p53-independent induction of p21 are abundant (see Review of the literature), only a few reports have connected it to UV radiation. p53 independency of p21 promoter activity was demonstrated by two ways: firstly, p21 promoter activity was found to be induced by UV in p53-/- cells, and secondly, deletion constructs devoid of both p53 binding sites showed normal promoter activity in NIH3T3 cells carrying normal p53 (III).

The induction of p21 by ionizing radiation, drugs, and other agents that cause double-strand breaks depends on intact p53 (Dulic et al., 1994; El-Deiry et al., 1994; Macleod et al., 1995). While UVB/A radiation has been shown to enhance p21 expression only in the presence of p53 (Liu and Pelling, 1995), UVC has been able to induce p21 protein in Li-Fraumeni cells (Loignon et al., 1997), in melanoma cells expressing mutant p53 (IV), and in p53-/- fibroblasts (III). Additionally, our study extended the findings to the transcriptional level, and was the first to determine the p21 promoter elements involved in p21 regulation after UV treatment (III). However, in contrast to our findings, UVC was recently found to be unable to induce p21 protein or mRNA in p53-/- MEFs, and, additionally, p21 induction in normal cells occurred through stabilization of p21 mRNA (Gorospe et al., 1998). Although Gorospe et al. (1998) failed to observe elevation of p21 protein levels in p53-/- cells, p21 mRNA was induced 3-4 fold, as compared to 25-fold increase in p53+/+ MEFs, the authors disregarded this relatively low level of induction in p53-/- cells. In our experiments both cell types showed equally strong p21 mRNA induction (maximum fold 5.2 and 6.7 in +/+ and -/- cells, respectively). Moreover, using the same p21 promoter luciferase construct, the authors observed only about 2-fold induction in luciferase activity while in our study the induction was 5-fold, but, most importantly, the induction was the same in -/- and +/+ cells (Gorospe et al., 1998). So, due to high p21 mRNA induction and low p21 promoter activity in normal cells the authors concluded that p21 induction resulted from stabilization of p21 mRNA rather than transcriptional activation (Gorospe et al., 1998). The stabilization of p21 mRNA is not an unusual phenomenon (Akashi et al., 1999), even if the regulation of the stability of mRNA is poorly understood. Of course, it cannot be ruled out that mRNA stabilization may participate in p21 induction in our cells as well, although p21 mRNA and promoter activity were equally induced (III); studies determining the halflife of mRNA would illuminate this aspect.

The regulatory element in the p21 promoter that totally abolished the UV response when absent was mapped between -110 bp and -61 bp proximal of the transcription start and was linked to two Sp1 binding sites (sites 2 and 4) within the region (III). This area seems to be of special importance in transcriptional activation of p21 promoter, since numerous p21 inducers, including TGF-ß (Datto et al., 1995), progesterone (Owen et al., 1998), okadaic and phorbol ester (Biggs et al., 1996) , nerve growth factor (Yan and

Ziff, 1997) and ras (Kivinen et al., 1999) are regulated by these same or neighboring Sp1-binding sites. A negative regulatory element -2.0 kb distal relative to transcription initiation site was found in NIH3T3 but not in p53-/- cells suggesting also a p53-mediated negative regulation of p21 promoter activity.

In this study UV-induced p21 expression paralleled growth arrest kinetics, its role as proliferation controller being supported by a rapid response regulated at transcriptional level both in the presence and absence of p53 (III). Accordingly, flavone-induced G1 growth arrest induced by flavone was shown to depend on p53-independent transcriptional activation of p21 (Bai et al., 1998). It is possible that in the absence of p53 p21 may in part compensate for the function otherwise accomplished by p53. On the other hand, it has been assumed that despite p53 independent regulation, p21 induction may be enhanced by p53. For example, PMA was shown to induce p21 independent of p53 but the induction was increased manyfold in the presence of p53 (Akashi et al., 1999) . For these reasons it was surprising that p21 was not only induced in the absence of p53, but that the level of protein and mRNA induction was completely independent of p53. However, two events were affected by a lack of p53: firstly, p53-/- cells were not able to reenter the cell cycle and many underwent apoptosis possibly reflecting deficient repair, and secondly, high UVC doses (50 J/m^2) caused attenuated induction of p21 protein. These findings suggest that p53 may be needed for full p21 induction after massive DNA damage and that p21 may function as a preventor of apoptosis.

The main results of UV-radiation induced activities of p53, pRB, and p21 in mouse fibroblasts, particularly their cell cycle phase and replication dependence, are summarized in the following figure.

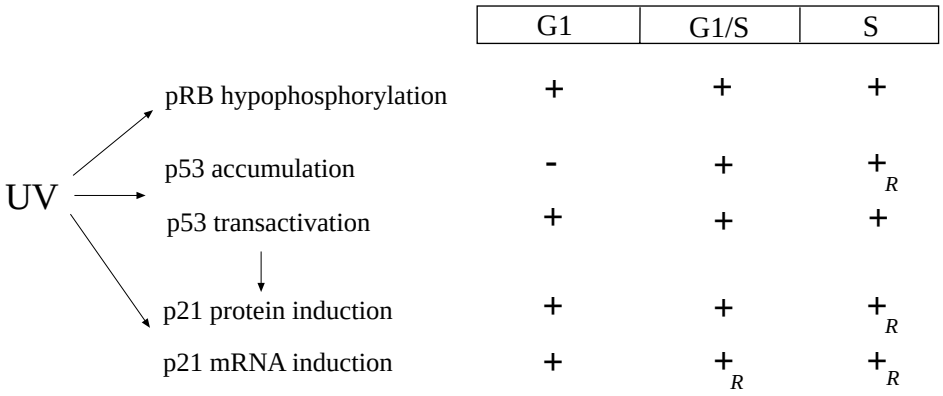

Fig. 6. Activity of pRB, p53, and p21 in different phases of the cell cycle induced by UV radiation. *R* indicates that the activity is dependent on DNA replication. Induction of p21 can occur directly by UV, but is probably augmented by p53 function.

Aberrant p53 functions in UV-treated melanoma cell lines (IV)

The low frequency of p53 mutations (Hartmann et al., 1996) and high basal expression of wild-type p53 often encountered in melanomas (Lassam et al., 1993; Floerens et al., 1994; Floerens et al., 1995) aroused our interest in exploring the UVC response of seven melanoma cell lines. Based on previous reports (Montano et al., 1994; Bae et al., 1996) as well as on our analysis of all p53 coding exons by RT-PCR and direct sequencing (IV), four melanoma cell lines (A-375, Malme-3M, WM239, and G361) appeared to harbor wild-type p53 and three cell lines mutant p53, including a nonsense mutation leading to a truncated p53 protein (RPMI-7951), and two mutations in p53 DNA-binding domain (SK-MEL-2, SK-MEL-28). Compared to melanocytes expressing low or negligible levels of p53 protein, all untreated melanoma cell lines, regardless of the p53 status, expressed high levels of p53 protein with the exception of RPMI-7951 which lacked detectable p53. Accumulation of both wild-type and mutant p53 was detected by western blotting and immunostaining of fixed cells, the latter revealing also the nuclear localization of p53 protein. p21 protein was expressed only in melanoma cells with wild-type p53, but its level failed to correlate with that of p53. Similarly, although mdm2 mRNA levels on the average were higher in cells expressing wild-type than in cells expressing mutant p53, high mdm2 levels were not associated with high p53 protein levels in individual melanoma cell lines (IV).

UVC treatment of 50 J/m^2 resulted in further accumulation of p53 only in wild-type cell lines. Although the kinetics of p53 stabilization in melanoma cell lines was consistent with that in NIH3T3 cells being detectable within 6 h incubation, the elevation in most wild-type cell lines was quite modest and less than in melanocytes. As previously shown (I), mutant p53 was not subject to further stabilization (IV).

To demonstrate transcriptional activity of p53, UV-responsiveness of three p53 target genes was examined. Induction of p21 protein was observed only in melanoma cell lines expressing normal p53, whereas induction of p21 mRNA occurred also in one mutant cell line (SK-MEL-28) lacking an increase in p21 protein. Interestingly, p21 protein and mRNA levels were not elevated until 16 to 24 h after exposure to UV reflecting a slower kinetics than seen in human and mouse fibroblasts (I, II). The GADD45 response, on the other hand, was rapid and simultaneous with growth arrest. GADD45 mRNA induction was detected in all UV-treated melanoma cells studied, maximal fold induction ranging from 2.8 to 8.8 independent of p53 status and p21 induction pattern. Moreover, high induction of GADD45 mRNA (4-fold or more) correlated with prominent apoptosis, which, like growth arrest, occurred irrespective of p53 status (IV). The UV response of mdm2 mRNA was consistent in all melanoma cells, with the exception of RPMI-7951: an initial decrease in mdm2 levels 6 h after UV

was followed by slight increase 24 h after UV treatment. In RPMI-7951 cells this decrease was absent and maximal induction took place already 16 h after UV. The inductions were small, and no significant difference was detected between cells expressing wild-type or mutant p53. Thus, regulation of target gene inductions by UV – though different from each other - appeared to be independent of p53 function (IV).

Since DNA damage can stimulate p53 effector genes also in a p53-independent manner, EMSA analysis was undertaken to examine the UV-induced specific DNA-binding activity of p53. Mutant cell lines were also studied in order to find out whether mutant p53 could induce p21 mRNA expression detected in SK-MEL-28. The specificity of binding of p53 to DNA was confirmed by using oligonucleotides containing specific p53-binding sites from either p21 or GADD45 promoters and by detecting supershifts of DNA-p53-antibody -complexes in the presence of p53 antibodies. As expected, UV-stimulated DNA binding activity of p53 to both p21 and GADD45 probes was detected in A-375, Malme-3M, and WM239 cell lines expressing wild-type p53 and was absent in SK-MEL-28, SK-MEL-2, and RPMI-7951 cells carrying mutant p53 (IV; data not shown). Surprisingly, however, G361 cells exhibiting high levels of wild-type p53 completely lacked UV-stimulated DNA-binding activity with all probes even in the presence of the antibody PAb421 that is known to stimulate the binding. This suggests that the UV response in G361 occurs independent of p53, or at least independent of its transactivation property. It should be also noted that when observed, DNA-binding activity was stimulated already 6 h after DNA damage thus hardly contributing to p21 induction detected only after 16 h incubation (IV).

The expression of some other cell cycle regulators, that are known to be involved in the pathogenesis of malignant melanoma, were also studied. pRB expression was observed in all melanoma cell lines, whereas only cell lines carrying mutant p53 showed p16 protein and mRNA expression (unpublished observation); the lack of p16 expression in wild-type p53 cell lines is due to premature stop codon in A-375 (Ohta et al., 1994), or deletion of exon α in WM239 and G361 cell lines (Ohta et al., 1994). In addition to the p53 mutation, SK-MEL-28 cell line harbors Cdk4 mutation (Tsao et al., 1998). It can be speculated that in the absence of p53 mutation, inactivating mutations of p16 or other targets of RB pathway have been selected.

Human melanoma represents one of the few malignancies that seldom select for p53 mutations (Hartmann et al., 1996). Although primary melanomas rarely contain p53 mutations, they are more frequently detected in melanoma cell lines (Albino et al., 1994; Bae et al., 1996) corresponding to mutation frequency of 43% among our seven randomly selected melanoma cell lines. On the other hand, all cell lines expressing wild-type p53 showed abnormal stabilization of normal p53. Accumulation of wild-type p53 was neither due to extranuclear localization nor due to overexpression of mdm2,

since mdm2 levels failed to systematically accompany p53 accumulation although they were generally higher in the wild-type than in mutant p53 cells (III, see also " Review of the literature"). Similarly, although all untreated melanoma cell lines harboring normal but stabilized p53 were expressing p21 protein, the levels of p53 and p21 did not correlate (IV). This is in contrast to non-Hodgkin´s lymphomas where overexpression of wild-type p53 was associated with accumulation of p21 (Maestro et al., 1997). Teratocarcinomas, which never contain p53 mutations, also exhibit high levels of nuclear wild-type p53. This protein is transcriptionally inactive until activated by DNA damage or by agents inducing differentiation which leads to transcriptional induction of target genes and p53-mediated apoptosis (Lutzker and Levine, 1996).

Our results indicate that despite the wild-type sequence of p53 its response to UVC radiation differed in several aspects from that of normally functioning p53. Irrespective of this, or due to it, the response patterns of mutant and wild-type p53 expressing melanoma cell lines did not show any major differences. Although wild-type p53 accumulated in response to UV as expected, the p53 pathway downstream of accumulation displayed several specific features: induction of p21 protein and mRNA expressions by UV radiation were delayed, 2) inductions of the target genes p21, GADD45, and mdm2 were dissociated from p53 regulation, 3) one wild-type p53 melanoma cell line had no UV-stimulated DNA-binding activity, and 4) of the cell cycle regulators studied only GADD45 expression correlated with growth arrest and apoptosis.

UVC-stimulated p21 protein and mRNA inductions appeared to be independent of p53 function, as accumulation and DNA-binding of p53 took place considerably earlier than p21 induction, and p21 mRNA elevation occurred with the same kinetics also in SK-MEL-28 cell line harboring mutant p53. However, p53 may still play a role in the full induction of p21, since p21 protein was not induced in SK-MEL-28 cells despite an elevation in the level of mRNA encoding it. This finding is consistent with the fact that p53-/- MEFs, but not p53+/+ MEFs, had an attenuated p21 protein response to high UVC dose (III), providing evidence that p53 activity is required for some aspects of p21 regulation. The requirement of p53 for growth arrest and p21 induction is certainly affected by the nature of DNA damage. Polyamine analogs usually cause G1 and G2/M arrests in p53 proficient cells, but treatment of SK-MEL-28 cells carrying mutant p53 have been shown to abolish G1 arrest and p21 induction (Kramer et al., 1999).

In contrast to stimulation of p21 expression, GADD45 mRNA induction occurred rapidly within 6 h after UV treatment in all melanoma cell lines irrespective of p53 status. p53-independent activation of GADD45 in response to UV has been detected previously (Kearsey et al., 1995). Since activation of GADD45, and not of p53, correlated with both growth arrest and apoptosis, GADD45 appeared to serve as a rapid

UV response target (IV). Contrary to our results, however, GADD45 has been proposed to protect cells from UV-induced apoptosis (Smith et al., 1996). Interestingly, GADD45 induction in γ-radiated melanoma cells expressing wild-type, accumulated p53 was very different from that after UV radiation. Ionizing radiation resulted in strong G1 growth arrest, prolonged p21 induction, and low or absent GADD45 induction (Bae et al., 1996). Taken together this suggests that GADD45 activation would be vital in UV response and regulated independent of p53, whereas it can be neglected after ionizing radiation and is probably under the control of intact p53.

Of the p53 effector genes mdm2 has recently received most interest due to its role as a negative regulator of p53, a function which is affected by several different p53 activating pathways. The regulation of mdm2 by UV radiation elicits some special features. Compared to p53 accumulation, low UV doses lead to rapid mdm2 induction, while with high UV doses mdm2 response is delayed, the decrease being p53-independent and the increase p53-dependent (Perry et al., 1993; Wu and Levine, 1997). The mdm2 response of melanoma cells studied here seemed to follow that pattern, but the increase in mdm2 mRNA levels after an initial decrease was detected also in two mutant p53 cell lines, one of them (RPMI-7951) displaying the highest mdm2 induction of all. Thus, UV-stimulated induction of mdm2, as well as that of p21 and GADD45, appeared to be independent of functional p53.

Although accumulation and DNA-binding activity of p53 were upregulated with rapid kinetics in all melanoma cell lines expressing wild-type p53 except for G361, none of the UV responses - growth arrest, apoptosis, or target gene inductions of p21, GADD45, or mdm2 - appeared to be regulated by p53. Despite the DNA-binding activity of p53 observed in the majority of cell lines, this suggests that abnormally stabilized wild-type p53 in melanoma cell lines is functionally inactive or possesses alternative functions not characterized here. The functional inactivity downstream of sequence-specific DNA binding would explain tolerance to high p53 levels and why mdm2 levels are not raised to downregulate p53. As in the case of p53 mutation, a cell with inactive p53 is not able to sense the effects of p53 and responds by decreasing p53 degradation leading to accumulation of inactive, though wild-type, p53 protein.

CONCLUSIONS

This study was undertaken to clarify how p53, presumably the best known cell cycle regulator and tumor suppressor, is regulated in transient growth arrest triggered by UV light. The overall cellular response to UV radiation was in many respects different from that shown to ionizing radiation, but some of the findings that at first appeared surprising have later been supported by others.

Unlike ionizing radiation, UV response studied in multiple cell lines was not abrogated by the absence of p53. Even during G1-phase growth arrest, the area believed to be governed by p53, pRB hypophosphorylation seemed to be at least equally important as activation of p53. While UV-induced transactivation of p53 was rapid taking place in all the cell cycle phases, accumulation of p53 seemed to correlate with replication of damaged DNA and, supposedly, the existence of unrepaired DNA lesions.

Regardless of apparently normal p53 status no melanoma cell line displayed normal p53 function. The identical growth arrest pattern in cell lines expressing mutant and wild-type p53 suggests no role for p53 in melanomas, whereas the functional inactivation of wild-type p53 indicates a loss of p53 function in melanomas. UV-triggered p21 induction was found to be regulated at the transcriptional level also in the absence of p53, but dissociation of p21 mRNA and protein expression was detected in several situations, and in some of them a defective p21 response was clearly caused by lack of functional p53.

Dissociation of stabilization and transcriptional activities renders p53 even more interesting, since accumulation may be involved in tasks other than those requiring transcriptional activity. Since these findings thus far apply only to mouse fibroblasts, it would be of particular interest to study the cell cycle phase-specific activation and phosphorylation of N-terminal residues of p53 in normal human fibroblasts. It would be also useful to explore whether induction of ARF, which represents a p53-activating pathway that is not induced by DNA damage, would result in different p53 responses than those detected after UV radiation.

UV radiation provides one model for damaging genetic material, and the knowledge gathered from cells treated with both UV and γ-irradiation is indicative of how damaged cells respond to and survive the DNA damage. Cell cycle phase studies like those presented in this thesis may determine in which phases p53 is indispensable and in which phases, and to what extent, its functions in damage response can be replaced by other cell cycle regulators, for example pRB or CKIs.

Along with advances in basic cancer research, the underlying mechanisms of the development or prevention of malignant transformation at the cellular level have become better understood. Instead of today´s often unspecific cancer treatment, novel

therapies can be developed in the future by taking advantage of the molecular level differences between normal and transformed cells. For example, the knowledge gained from the interplay between p53 and adenovirus proteins have yielded a clinical trial where promising results have been obtained by infecting carcinoma patients with mutant adenoviruses that cause cytopathic effect only in cells lacking functional p53. These kinds of trials, demanding wide-ranging expertise of many fields, lie at the very heart of medicine reaching from knowledge to action.

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