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STUDIES ON MODULATION OF THE ACTIVITY OF TUMOR SUPPRESSOR PROTEIN p53

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in Cell Biology) on May 18, 2004 by the council of the Institute of Molecular and Cell Biology, University of Tartu

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Commencement: June 28, 2004

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To my mother with love

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to by the relevant Roman numeral in the text:

I Joers, A., Kristjuhan, A., **Kadaja, L.**, and Maimets, T. 1998. Tumour associated mutants of p53 can inhibit transcriptional activity of p53 without heterooligomerization. Oncogene 17: 2351–8.

II Lepik, D., Jaks, V., **Kadaja, L.**, Varv, S., and Maimets, T. 2003. Electroporation and carrier DNA cause p53 activation, cell cycle arrest, and apoptosis. Anal Biochem 318: 52–59.

III **Kadaja, L.**, Laos, S., and Maimets, T. 2004. Overexpression of leukocyte marker CD43 causes activation of the tumor suppressor proteins p53 and ARF. Oncogene 23: 2523–2530.

ABBREVIATIONS

3'UTR	3' untranslated region
53BP1	p53-binding protein 1
53BP2	p53-binding protein 2
APAF-1	apoptosis protease-activating factor 1
APC	adenomatous polyposis coli
ARF	alternative reading frame
ASPP	apoptosis stimulating proteins
ATM	ataxia-telangiextasia mutated
ATR	ATM and Rad3 related
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma protein-2
Bcl-X	Bcl-2-like protein-X
Bid	BH3 interacting domain death agonist
BRCA1	breast cancer susceptibility gene 1
BrdU	5-bromo-2-deoxyuridine
CAK	cycline activating kinase
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
CD	cluster of differentiation
CDK	cyclin-dependent kinase
Chk	checkpoint kinase
CKI	casein kinase I
CKII	casein kinase II
CMV	cytomegalovirus
CON	p53 binding consensus DNA sequence
CREB	cyclic AMP-response element binding protein
C-terminus	carboxy-terminus
DNA-PK	DNA-dependent protein kinase
DR5/KILLER	death receptor 5
dTAFIIH40	TATA-binding-protein-associated factor II40 from *D. melanogaster*
dTAFIIH60	TATA-binding-protein-associated factor II60 from *D. melanogaster*
GADD45	growth arrest and DNA-damage inducible gene 45
GSK-3β	glycogen synthase kinase 3β
HDAC	histone deacetylase
HIPK2	homeodomain interacting protein
HMG-1	high mobility group protein 1
hTAFII31	human TATA-binding-protein-associated factor II31
iASPP	inhibitory member of the ASPP family
IGF	insuline-like growth factor

IGF-BP3	insuline-like growth factor-binding protein 3
ING1	inhibitor of growth factor 1
IR	ionizing radiation
JMY	junction-mediating and regulatory protein
JNK	c-Jun N-terminal kinase
kb	kilobasepairs
kDa	kilodalton
MAPK	mitogen activated protein kinase
MCF-7	mammary carcionoma female-7
Mdm2	mouse double minute 2
MEF	mouse embryonic fibroblasts
mSin3a	mammalian Sin3a
NES	nuclear export signal
NFκB	nuclear factor κB
NLS	nuclear localization signal
N-terminus	amino-terminus
P53AIP1	p53-regulated apoptosis-inducing gene 1
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PEI	polyethylenimine
PERP	p53 apoptosis effector related to PMP-22
PI3K	phosphatidylinositol 3 kinase
Pidd	p53-induced protein with a death domain
PIG3	p53-induced gene 3
PKB	protein kinase B
PKC	protein kinase C
PML	promyelocytic leukaemia
PP2AB	protein phosphatase 2AB
pRB	retinoblastoma protein
PTEN	phosphatase and tensin homolog
PUMA	p53 up regulated modulator of apoptosis
RPA	replication protein A
SH3	Src homology domain 3
ssDNA	single stranded DNA
TAF	TATA box-binding protein -associated factor
TBP	TATA box-binding protein
Tcf/Lef	T-cell factor/Leukocyte enhancing factor 1
TFIID	transcription factor II D
TFIIH	transcription factor II H
UV	ultraviolet
WAF1	wild type p53-activated fragment 1
WIP1	wild type p53-induced phosphatase
WISP1	wnt-1-induced secreted protein
WT1	Wilms' tumor protein 1

INTRODUCTION

Cancer is a serious disease caused by defective control of cell proliferation. The inactivation of tumor suppressor genes and deregulated expression of oncogenes is often the cause of cellular transformation. As a tumor cell divides, each daughter cell inherits the accumulating genetic defects, leading to tumor development with possible progression to malignancy. This has inspired the model of tumorigenesis as a multistep process, which first overcomes the primary safeguard against cellular transformation through additional changes, mainly inactivating the tumor suppressor genes. The identification of the genes linked with cancer is essential for the understanding of the regulation of cell proliferation and for the development of the therapeutic strategies to eliminate the cancer cells.

The most commonly affected gene in human cancer is tumor suppressor P53, which functions in the regulation of cell proliferation. Mutation of P53 or loss of its gene causes abnormal function of the cell and is involved in tumor development. This study deals with p53 protein, which is a sequence-specific DNA-binding protein that promotes cell-cycle arrest or apoptosis in response to a variety of cellular stresses, and is therefore aptly termed the ‘guardian of the genome’.

The main purpose of the present study was to investigate the activating and inactivating mechanisms, that modulate the activity of the p53 protein. The results of the study open new perspectives for the mechanism of inactivating the wild type p53 by its mutant counterparts. As well, as give the new insight for the abnormal expression of CD43 in solid tumors. During this study, it has been shown that the choice of a method for treating cells is important in the case of studying such a sensitive stress-protein as p53.

REVIEW OF LITERATURE

1. p53 – general introduction

p53 was discovered 25 years ago being first identified as a transformation-associated protein (DeLeo *et al.* 1979; Kress *et al.* 1979; Lane and Crawford 1979; Linzer and Levine 1979; Rotter *et al.* 1980). Within following 10 years, the researchers realized that they had studied the p53 mutants that are present with high frequency in human cancers. The wild type p53 protein, on the contrary, is a very important factor in tumor suppression. This explained the inconsistent data about this protein obtained during this period. p53 was therefore renamed for tumor suppressor and has become probably one of the most studied proteins ever since. This resulted in the enormous amount of information that has been gathered about p53 by now. p53 turned out to be a nuclear multifunctional phosphoprotein with characteristics of a transcription factor that controls genomic plasticity and integrity, being involved in a wide range of cellular processes.

The p53 gene is a single copy gene and localizes in the 17th chromosome in human and in the 11th chromosome in mouse. Its size is about 20kb containing 11 exons (Bienz *et al.* 1984; Rotter *et al.* 1984; Benchimol *et al.* 1985; Lamb and Crawford 1986). The length of the p53 mRNA is 2.8 kb and 2 kb in human and mouse, respectively (Matlashewski *et al.* 1984; Harlow *et al.* 1985; Zakut-Houri *et al.* 1985).

Activities that have been attributed to p53 are as follows: regulation of gene expression (both activation and repression), DNA synthesis and repair (Ford and Hanawalt 1995; Wang *et al.* 1995; Li *et al.* 1996), control of DNA replication (Cox *et al.* 1995), DNA damage response and cell cycle control. p53 acts also in cellular differentiation (Rotter *et al.* 1994), senescence (Wynford-Thomas 1999), inhibition of angiogenesis (Bouck 1996), and in programmed cell death (Levine *et al.* 1991; Rotter and Prokocimer 1991; Montenarh 1992; Oren 1992; Vogelstein and Kinzler 1992). p53 has gained special interest due to its activation in response to cellular stress to mediate a variety of anti-proliferative processes. p53 protein is a sensor of diverse forms of stress such as genotoxic stress (UV and IR, cytotoxic drugs, carcinogens), various non-genotoxic stresses (hypoxia, temperature changes, redox changes) and oncogenic stress (Ko and Prives 1996; Levine 1997). Disruption of p53 function promotes checkpoint defects, cellular immortalization, genomic instability, and appropriate survival, allowing the continued proliferation and evolution of damaged cells (Hollstein *et al.* 1991; Hainaut *et al.* 1998).

It is well accepted that the inactivation of wild type p53 is a key event in tumor development. Evidence that p53 is a tumor suppressor came from genetic studies showing that p53-deficient mice develop normally but are prone to

tumors with high frequency by 6 months of age (Donehower *et al.* 1992). The observation that p53-deficient mice develop mostly normally was somewhat surprising, because p53 was believed so far to be essential not only for tumor suppression but also for normal development. The normal development of p53-deficient mice could be explained with the later discovery of the new p53 family members, p63 and p73 (Lohrum and Vousden 2000). In contrast to p53, neither p73 nor p63 appears to be frequently mutated in human cancer, which means that they seem not to be critical for tumor suppression. Unlike to p53-deficient mice, mice lacking p63 and p73 do not develop tumors, but have significant developmental abnormalities instead. Thus, rather p63 and p73 than p53, have a role in normal development (Donehower *et al.* 1992; Irwin and Kaelin 2001). p73 and p63 have striking homology to p53, the difference occurs at the C-terminus, whereas p63 and p73 are more similar to each other than to p53 (Chen 1999; Kaelin 1999). p63 and p73 are required for p53-dependent apoptosis in response to DNA-damage, but can also induce cell death independently of p53 (Lissy *et al.* 2000; Stiewe and Putzer 2000; Flores *et al.* 2002).

Taken together, p53 seems not to have an absolute role in tumor development as believed so far, and the complexity of the story around this pretentious protein increases with each new publication. Due to the huge amount of information about p53 only the aspects most related to present studies including the structure of p53, mechanisms for modulation of its activity and the main roles of this protein in tumor suppression, will be discussed more thoroughly.

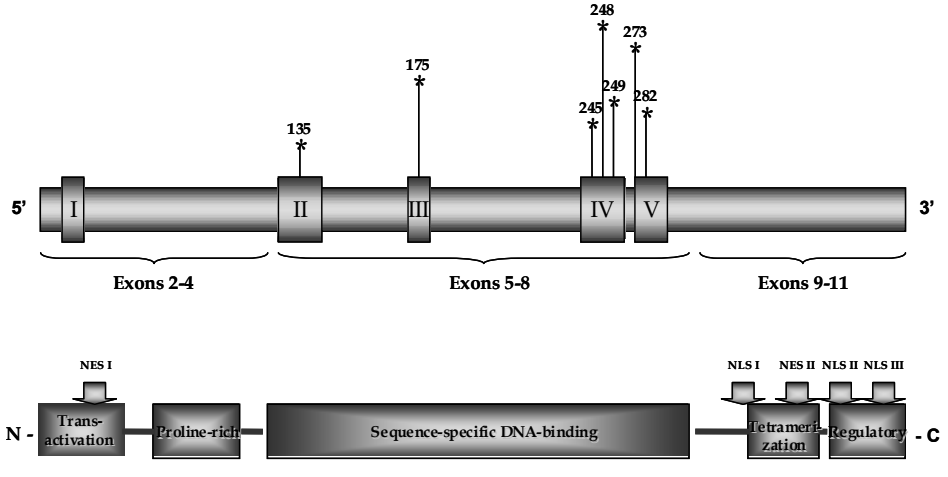

Figure 1. Schematic representation of the p53 gene and protein. The upper panel represents the p53 gene. The five conserved regions are given as Roman numerals and the regions of exons 2–11 as Arabic numerals (the first exon is non-coding; (Bienz-Tadmor *et al.* 1985). * represent the number of most frequently mutated codons in human tumors (Cariello *et al.* 1994). Lower panel represent functional domains of the p53 protein. NLS – nuclear localization signal, NES – nuclear export signal. N- and -C are amino-terminus and carboxy-terminus, respectively.

2. Structural and functional domains of the p53 protein

The human p53 protein consists of 393 amino acids and its apparent molecular weight is ~53 kDa (Harlow *et al.* 1985; Zakut-Houri *et al.* 1985). The protein can be divided into several functional domains including transcriptional activation domain at the amino-terminus (amino acids 1–42), sequence-specific DNA binding region within the central part (amino acids 102–292), oligomerization domain (amino acids 360–393), and a regulatory domain (amino acids 360–393) in the carboxy-terminal region (May and May 1999). The p53 gene contains five highly conserved regions, termed domains I–V (correspond with amino acids 13–23, 117–142, 171–181, 234–258, and 270–286) which coincide with the regions crucial for the p53 functions (Soussi *et al.* 1987; Soussi *et al.* 1990; Soussi and May 1996) (Figure 1).

2.1. The amino terminal region

The first 42 amino acids of p53 make up an acidic region called transactivation domain, because this region regulates gene expression via binding with basal transcription machinery components (Lu and Levine 1995; Thut *et al.* 1995; Wang *et al.* 1995). Later, second transcripton activation domain has been described (between amino acids 43 and 73) (Venot *et al.* 1999). p53 has a proline-rich region (amino acids 63–97) with similarity to SH3 (Src homology domain 3)-binding proteins that is situated between the transactivation and DNA binding domains. This region is necessary for transcriptional repression by p53 and is required for (transcription-independent) growth suppression by p53-mediated apoptosis (Walker and Levine 1996; Sakamuro *et al.* 1997; Venot *et al.* 1998). The activity of p53 is modulated by the interaction of the N-terminal part with other proteins and several posttranslational modifications take place in this part of p53.

2.2. The ‘core’ domain

The DNA-binding domain or ‘core’ domain spans from amino acid 102 to 292 and forms a separate independently folding structure that binds to the DNA sequence specifically (El-Deiry *et al.* 1992; Bargonetti *et al.* 1993; Halazonetis and Kandil 1993; Pavletich *et al.* 1993; Wang *et al.* 1994). In human tumors, the p53 protein is often mutated and mutant proteins have principally lost the growth suppression functions. 95% of tumor-related mutations map to the residues of the DNA binding region and among them certain ‘hot spots’ of mutations have been described. These mutations occur at residues essential for DNA-binding and therefore inactivate the transcriptional activation function of

p53, giving an example how p53 is inactivated in tumors (Levine *et al.* 1991; Kern *et al.* 1992). The region in the core domain contains also region necessary for the binding of p53 with the negative regulators of apoptosis, Bcl-X_L and Bcl-2 (Mihara *et al.* 2003). Altogether, these findings confirm how important this region is for the function of p53 as a tumor suppressor.

2.3. The C-terminal region

The central 'core' domain is connected to the C-terminal region with the flexible linker region (amino acids 300–318) (Arrowsmith and Morin 1996). p53 requires nuclear localization for the function (Shaulsky *et al.* 1991a; Shaulsky *et al.* 1991b), which is ensured by three nuclear localization sequences in the C-terminal region (amino acids 316–325, 369–375, 379–384) (Dang and Lee 1989; Shaulsky *et al.* 1990b). The p53 export from the nucleus has been shown to be mediated by two nuclear export signal sequences located in the activation domain (amino acids 11–27) and in the tetramerization domain (amino acids 339–352) of p53 (Stommel *et al.* 1999; Zhang and Xiong 2001). Full length p53 protein forms stable tetramers and the tetramerization domain including amino acids 324–355 is responsible for the oligomerization of p53 (Arrowsmith and Morin 1996). Although monomeric p53 is able to bind DNA, activate transcription, and suppress growth (Friedman *et al.* 1993; Shaulian *et al.* 1993; Slingerland *et al.* 1993; Tarunina and Jenkins 1993), p53 is believed to function much more efficiently as a tetramer probably due to the greater DNA binding affinity (Shaulian *et al.* 1993; Jeffrey *et al.* 1995; Pellegata *et al.* 1995).

Studies on the regulation of p53 tertiary structure have provided ideas for the conformation model for the functioning of p53. According to this model, mutations that deregulate the normal control of p53 conformation may lead to cancer (Milner 1991; Milner 1994). It has been shown that if one of the two alleles of the p53 gene is mutated in the cell, the mutant p53 protein can inactivate the wild type p53 in a dominant-negative manner. This dominant-negative activity has been explained with the ability of the mutant p53 protein having different conformation to form mixed tetramers with wild type p53 driving the latter into the 'mutant conformation' (Milner *et al.* 1991; Milner and Medcalf 1991). These kinds of hetero-oligomers lack the growth suppression function (Kern *et al.* 1992). Due to such dominant negative effect over wild type protein, the wild type p53 cannot avoid malignant growth if it co-expresses with the mutant p53 protein. In addition to the loss of wild type p53 function, some mutant proteins appear to have new functions contributing to transformation and tumor development (Dittmer *et al.* 1993). Thus, the conformational organization of the p53 molecule has important role for the proper function.

Next to the oligomerization domain is the basic region (amino acids 363–393), named regulatory domain, which is required for regulation of the p53

activity. p53 is unusual among transcription factors, because it has also non-specific DNA-binding ability in addition to the sequence-specific DNA-binding. The basic region of p53 binds with high affinity to single-stranded DNA (ssDNA), gamma-irradiated DNA, Holliday junctions, stem-loops, insertion or deletion mismatches and recombination intermediates. All these events have been shown to activate p53 sequence-specific DNA binding (Ahn and Prives 2001). Modifications of these 30 amino acids including phosphorylation (Prives 1998) and acetylation (Gu and Roeder 1997; Liu *et al.* 1999), or proteolytic removal (Hupp *et al.* 1992; Okorokov *et al.* 1997) can promote the p53-dependent transcriptional induction. In addition, glycosylation (Shaw *et al.* 1996) and RNA binding (Cassiday and Maher 2002) have also been observed during p53 activation.

A model for the activation of p53 has been proposed according to which the C-terminus of p53 interacts with the core domain. This interaction locks the core domain into a conformation keeping p53 in a latent, low-affinity DNA-binding form that is inactive for DNA binding (Hupp *et al.* 1995). The core domain is able to adopt an active conformation (efficient DNA-binding) after the modification or deletion of C-terminus, or protein-protein interaction. The binding of ssDNA ends or short ssDNA fragments to C-terminal domain may also stabilize p53 in a conformation, active for binding to target DNA sequences (Yakovleva *et al.* 2001). Furthermore, the proline-rich region at the N-terminus of p53 has been suggested to cooperate with C-terminus for the maintenance of the latent, low-affinity DNA binding conformation of p53 (Müller-Tiemann *et al.* 1998). Thus, p53 requires a structural change for the activation of sequence specific DNA binding and this occurs through both N-terminal region and the basic C-terminal domain.

3. Modulation of the activity of p53

The p53 protein remains passive in the cell under normal conditions, probably by existing in a latent form unable to activate transcription and requiring modifications to become active. As p53 is a potent inhibitor of cell growth, its function must be tightly controlled to allow normal growth and it is activated only at need. This is achieved through several mechanisms that include p53 gene transcription and translation of p53, control of protein stability and sub-cellular localization. In addition to the accumulation of the p53 protein, the mechanisms including the protein-protein interactions and post-translational modifications are also necessary for the maximal functional activity of p53 as a transcription factor.

3.1. Transcriptional and translational control

Regulation of p53 at the transcriptional level is not common and that is not surprising, because it would be disadvantageous to express such an important control factor after the damage of DNA template. More propitious is the regulation at the level of translation that enables to modulate the p53 function in an extremely rapid manner in most physiological situations, if needed. In unstimulated cells, translation of human p53 mRNA is repressed constitutively by its 3' untranslated region (UTR)(Fu *et al.* 1996). This is mediated by p53 itself through binding to its own mRNA and inhibiting translation. This translational repression is relieved upon exposure to DNA damage, for example (Mosner *et al.* 1995). Thus, the inhibition of p53 biosynthesis requires wild-type p53 itself.

3.2. Control of the p53 protein half-life

The p53 protein level in the cell is determined by the rates of its synthesis and degradation. p53 has a short half-life of only about 10 minutes in normal (nontransformed) cells due to rapid degradation (Ashcroft *et al.* 1999). The rapid turnover of the p53 protein is mediated by Mdm2 oncogene (Hdm2 in humans), the key regulator of p53. Mdm2 functions in two ways: Firstly, Mdm2 induces the ubiquitin-dependent proteolytic degradation of p53 (Haupt *et al.* 1997; Honda *et al.* 1997; Kubbutat *et al.* 1998). Secondly, Mdm2 binds to the N-terminus of p53 (amino acids 18–23) and masks the transactivation domain of p53 thus inhibiting the p53-mediated transactivation (Momand *et al.* 1992; Chen *et al.* 1993; Oliner *et al.* 1993; Picksley *et al.* 1994). The activated p53 induces the MDM2 gene expression, and the Mdm-2 protein regulates the p53 protein at the level of its activity. This results in negative feedback control of p53 activity (Wu *et al.* 1993). As Mdm2 regulates negatively the transcriptional activation of the p53 protein, this feedback mechanism is abrogated during cellular stress such as DNA damage (Shieh *et al.* 1997), activation of oncogenes (de Stanchina *et al.* 1998; Zindy *et al.* 1998) and hypoxia (An *et al.* 1998). Several mechanisms influence the interaction between p53 and Mdm2, resulting either in the stabilization and activation of the p53 protein or, on the contrary, inactivation and even enhanced degradation of p53, depending on which signaling pathway is prevalent.

3.3. Post-translational modifications that alter the functional activity of p53

Determination of the necessity of the functional domains of p53 was merely the beginning of understanding the modulation of its activity. The story of the complexity of the regulation of p53 activity continues with the discovery and description of different post-translational modifications such as phosphorylation, acetylation, and glycosylation that determine the character of the p53 response. Distinct stimuli lead to different posttranslational modifications on p53 (Kapoor and Lozano 1998; Lu *et al.* 1998; Webley *et al.* 2000). It is possible that p53 molecules with distinct modifications may have different promoter preferences or recruit distinct transcriptional coactivators, thus leading to the activation of a distinct population of p53 target genes and different cellular response.

Phosphorylation of p53. Several protein kinases can phosphorylate p53 in distinct sites in both the amino- and the carboxy-terminal domains (Figure 2) (Giaccia and Kastan 1998; Bulavin *et al.* 1999; Meek 1999; Prives and Hall 1999; Turenne and Price 2001). Phosphorylation of p53 affects the transactivation function of p53. This can be achieved through modulating Mdm2-p53 interaction and/or increased binding to a coactivator protein (Shieh *et al.* 1997; Dumaz and Meek 1999; Yuan *et al.* 1999). At present, the exact role of the each modification is not clear. For example, on the one hand both serine 15 and 20 have been shown to be critical in modulating the negative regulation of p53 by Mdm2 (Unger *et al.* 1999; Hirao *et al.* 2000; Shieh *et al.* 2000). On the other hand, there is evidence that p53 can be stabilized also without phsphorylation of these residues (Kubbutat *et al.* 1997; Ashcroft *et al.* 1999). These contradictory data suggest that the phosphorylation events depend largely on the cell type and stress stimulus.

Phosphorylation of serine 46 of human p53 has been shown to be necessary for the activation of some apoptosis-inducing genes (Fridman and Lowe 2003). Homeodomain-interacting protein kinase-2 (HIPK2) co-localizes with p53 and CREB-binding protein (CBP) within promyelocytic leukaemia (PML) nuclear bodies that have been postulated to serve a ‘meeting place’ for p53 and p53-interacting proteins. Several post-translational modifications, critical for its function, occur in the PML bodies. HIPK2 binds to and activates p53 by directly phosphorylating it at serine 46 in response to ultraviolet radiation (D'Orazi *et al.* 2002; Hofmann *et al.* 2002). Phosphorylation of p53 at serine 46 facilitates the CBP-mediated acetylation of p53 at lysine 382 via the formation of trimeric p53-PML-CBP/p300 complex, promoting p53-dependent gene expression, leading to enhanced p53-mediated apoptosis (Hofmann *et al.* 2002).

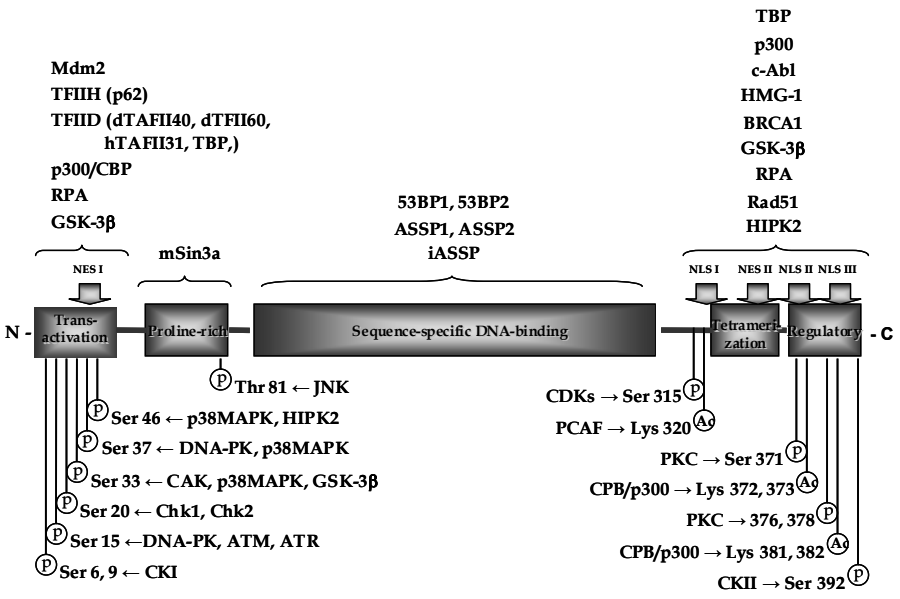

Figure 2. p53 and the accessory proteins. Sites for posttranslational modifications and modifiers are indicated (Ser – serine, Thr – threonine, Lys – lysine; P – phosphorylation site, Ac – acetylation site).

Acetylation of p53. Acetylation of the lysines at the carboxy-terminus of p53 is mediated by histone acetyl-transferases, such as CBP/p300 and PCAF (p300/CBP-assocoated factor) (Gu and Roeder 1997). CBP/p300 bind with the amino-terminus of p53 and can both positively and negatively regulate p53 transactivation. Firstly, CBP/p300 perform a binding platform to allow the assembly of the protein complex necessary for Mdm2-mediated degradation of p53 (Grossman *et al.* 1998). Second role for CBP/p300 is to act as the coactivator for p53-mediated transcription that interacts with p53 through its N-terminus, stabilizes it, and enhances the transcriptional activity of p53 (Avantaggiati *et al.* 1997; Lill *et al.* 1997; Thomas and White 1998). p300/CBP-mediated acetylation and activation of p53 is inhibited by Mdm2 (Kobet *et al.* 2000). Tumor suppressor ARF in turn prevents this inhibition of p53 acetylation by Mdm2 (Ito *et al.* 2001). CBP/p300 are complexed with PCAF (Yang *et al.* 1996). CBP/p300 acetylates lysines 372, 373, 381 and 382 in the NLSII and NLSIII at the carboxy-terminal region of p53 and PCAF acetylates lysines 320 within the linker region that connects central 'core' domain and C-terminal region (Sakaguchi *et al.* 1998; Prives and Manley 2001). All of these modifications have been proposed to induce the transcriptional activation function of p53, probably promoting the coactivator recruitment and histone acetylation (Barlev *et al.* 2001).

Other mechanisms for the modification of p53. p53 has also been shown to be O-glycosylated leading to the activation of p53 DNA binding (Shaw *et al.* 1996). In addition, the p53 protein can also be modified by sumoylation of certain lysine residues, and these changes may contribute to p53 activation (Meek 1999; Alarcon-Vargas and Ronai 2002). Whether these modifications qualitatively influence the outcome of p53 activation remains unclear.

Post-translational modifications seem to work in a coordinate manner, because some modifications influence the others. In other words, phosphorylation or acetylation of p53 at specific site may often favor the phosphorylation of p53 at another specific site. It is noteworthy that the post-translational modification sites described so far are mainly located in the regulatory regions of the p53 protein at the N- and C-terminus.

3.4. Intracellular localization of p53

It is clear that for DNA-binding and activation of transcription, p53 must be located in the nucleus. Relocation of p53 into the nucleus after cellular stress is desirable to inhibit cell growth of malignant cells. During the cell cycle p53 is differently located in the cell referring that the control of intracellular localization of p53 is cell cycle regulated (Shaulsky *et al.* 1990a). In certain types of tumors, the nuclear exclusion of wild-type p53 is often observed (Stommel *et al.* 1999), which is the additional mechanism of inactivating p53 function. Thus, appropriate sub-cellular localization is crucial for regulating the p53 function. p53 is actively transported through the nuclear membrane in both directions, and both nuclear import and export of p53 are tightly regulated (Vousden and Woude 2000; Ryan *et al.* 2001). p53 has both nuclear localization and nuclear export signals. Nuclear import of p53 is enabled by three nuclear localization signals (NLS) (Roth *et al.* 1998; Liang and Clarke 1999) while nuclear export is enabled by its two nuclear export signals (NES) (Stommel *et al.* 1999; Zhang and Xiong 2001). Several proteins influence nuclear import and export of p53 including p14ARF and c-Abl, which are positive regulators of p53 (O'Brate and Giannakakou 2003). Third mechanism for the inhibition of the p53 activity by Mdm2 is that Mdm2 binding can promote the export of p53 from the nucleus to the cytoplasm (Boyd *et al.* 2000; Geyer *et al.* 2000). In this case, it has been suggested that Mdm2-mediated ubiquitination activates or exposes a nuclear export signal in the p53 C-terminus, leading to the export of p53 from the nucleus to the cytoplasm.

Both in response to DNA-damage and to oncogene activation, p53 is recruited into nuclear bodies by tumour-suppressor protein PML resulting in p53 transactivation in a promoter-specific manner and induction of apoptosis (Fogal *et al.* 2000; Guo *et al.* 2000). However, in the case of Ras expression, the premature senescence is induced (Pearson *et al.* 2000).

It is generally believed that p53 induces apoptosis by target gene regulation and transcription independent signaling. Although the mechanism is not well understood, it has been shown that a fraction of the p53 protein in damaged cells translocates into mitochondria and that these cells duly undergo apoptosis and targeting p53 to mitochondria is sufficient to induce apoptosis (Marchenko *et al.* 2000; Moll and Zaika 2000; Sansome *et al.* 2001).

Subcellular localization of p53 has been reported to be regulated through interactions with the microtubule network. The nuclear localization of p53 requires the activity of microtubule-associated molecular motor and functional microtubules. The dynein motor proteins participate in transport of p53 and facilitate its accumulation in the nucleus after DNA damage. Transport along the microtubules has been observed only in response to stress and the interaction with microtubules is either directly or indirectly mediated by the amino-terminal region of p53 (Giannakakou *et al.* 2000).

3.5. Interactions of p53 with regulative proteins

p53 interacts with numerous cellular proteins that modulate its functional activities (Figure 2). These proteins act by different mechanisms with different outcomes like participating in the intracellular transport of p53, influencing the ability of p53 to bind DNA and activating transcription and affecting p53 half-life. For instance, Mdm2 in addition to inactivating p53 and inducing its degradation has also effects on the localization of the p53 protein as mentioned previously.

Both transcriptional activation and repression involve the direct interaction of p53 with the basal transcriptional machinery. The p53 protein activates transcription of a target gene by binding to a specific DNA response element and interacting with the basal transcription factor TFIIH from the transcriptional apparatus of RNA polymerase II (Wang *et al.* 1995). This event needs previous interaction of p53 with basal co-activators such as p300/CBP and PCAF that assure the maximal activity of p53 (Scolnick *et al.* 1997; Wadgaonkar *et al.* 1999; Espinosa and Emerson 2001). CBP/p300, acts through its interaction with the N-terminus of p53 and increases the sequence-specific DNA-binding activity of p53 by acetylating its C terminus (Yuan *et al.* 1999). The N-terminal activation domain of p53 interacts also with the components of the TFIID complex, such as dTAFII60 and dTAFII40, and hTAFII31 that mediate transcriptional control by p53 (Lu and Levine 1995; Thut *et al.* 1995). Mdm2 may repress transcriptional activation by displacing these proteins from p53, since these co-activators of p53 and Mdm2 share common binding site on p53 at the N-terminus (Lin *et al.* 1994). Both N- and C-terminal domains of p53 interact with another component of the TFIID complex, TATA-binding protein (TBP), which is required for transcriptional repression by p53 (Seto *et al.* 1992; Truant *et al.* 1993; Horikoshi *et al.* 1995).

Some p53-interacting proteins modulate overall p53 activity, but some have been shown to activate the expression of specific p53-inducible genes. For example, JMY (junction-mediating and regulatory protein) form complex with p300/CBP and p53 induce the p53-dependent transcription of the Bax gene and apoptosis (Shikama *et al.* 1999). Another example is the ASPP proteins (apoptosis stimulating proteins; ASSP1 and ASSP2) that interact with p53 and specifically enhance p53-induced apoptosis (Samuels-Lev *et al.* 2001). iASSP (inhibitory member of the ASPP family), on the contrary, acts as a inhibitor of the p53-dependent apoptosis through interaction with p53. Moreover, iASPP expression is upregulated in human breast carcinomas expressing wild-type p53 and iASPP cooperates with oncogenes to transform cells in vitro (Bergamaschi *et al.* 2003). Two p53-binding proteins, 53BP1 and 53BP2, bind to wild type p53 via the DNA-binding domain of p53 and enhance p53-mediated transcriptional activation and induce the expression of its target genes (Iwabuchi *et al.* 1994; Iwabuchi *et al.* 1998). c-Abl binds to the C-terminus of p53 after DNA damage, stabilizes the p53-DNA complex and enhances the transcriptional activity of p53 resulting in the block of the cell cycle progression (Nie *et al.* 2000). Chromatin-associated nucleoprotein HMG-1 (high mobility group protein-1), which bends DNA to facilitate the binding of various transcription factors to their cognate DNA sequences, binds to p53 and enhances its DNA binding (Jayaraman *et al.* 1998; Imamura *et al.* 2001). p33ING1 binds to and modulates p53-dependent transcriptional activation in cell growth control (Garkavtsev *et al.* 1998). Neither of p53 and p33ING1 can cause growth suppression when the other one is suppressed (Goga *et al.* 1995). The Wilms' tumor-suppressor gene product, WT1, has been shown to associate with the p53 protein and stabilize it. WT1 modulates the trans-activational properties of p53 inhibiting its ability to induce apoptosis (Maheswaran *et al.* 1993; Maheswaran *et al.* 1995). BRCA1 and p53 can cooperatively induce apoptosis of cancer cells. Tumor suppressor BRCA1 has been shown to interact with p53 and mediate p53-dependent transcriptional activation (Ouchi *et al.* 1998; Zhang *et al.* 1998a). Recently, the direct interaction between p53 and GSK-3β has been described (Watcharasit *et al.* 2003). GSK-3β has been shown to be required for the down regulation of β-catenin by p53 (Sadot *et al.* 2001).

These are only some examples of factors that bind to p53 and influence the role of p53 in the cell. The interaction between p53 and transcriptional co-activators has been suggested to influence its affinity for promoters. It is believed that the specific co-factors that are expressed in a particular cellular context determine the induction of specific group of p53-target genes. The decision whether the cell undergoes growth arrest or apoptosis, or even a particular apoptosis pathway, could also be done according to the availability of specific co-activators.

4. Functions of p53

Activation of p53 for tumor suppression has mainly two outcomes: cell growth arrest or apoptosis, which are mediated through a large number of genes being transcriptionally activated or repressed by p53.

The growth suppression activity mainly relies on its ability to interact with DNA sequence-specifically and activate transcription from promoters containing its binding site. p53 binding site consists of two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0–13 base pairs (Pu and Py are the purine and pyrimidine base-containing nucleotides, respectively) (El-Deiry *et al.* 1992; Funk *et al.* 1992). Several p53 target genes that mediate signals for cell cycle arrest and apoptosis including $p21^{WAF1/CIP1}$, MDM2, GADD45, BAX and IGF BP-3 contain p53 binding site in their promoters (Ko and Prives 1996).

p53 can also activate target genes through non-canonical sequence. The first such example is in the p53-induced gene 3 (PIG3), which has been implicated in the accumulation of reactive oxygen species and apoptosis induction (Polyak *et al.* 1997). p53 directly binds and activates the PIG3 promoter through a pentanucleotide microsatellite sequence within it (Contente *et al.* 2002). Another example is the pro-apoptotic phosphatase PAC1, which is induced under specific stress conditions and is necessary for p53-mediated apoptosis. p53 regulates transcription of PAC1 through binding to a novel palindromic binding site (Yin *et al.* 2003). These examples represent a novel mechanism for transcriptional regulation of apoptotic genes by p53, which differs from the classical one.

In addition to transactivation of different genes, p53 is also capable of repressing transcription, which has been shown not to require the transactivation activity of p53. p53 specifically represses the activity of promoters whose initiation is dependent on the presence of a TATA box and repression is mediated by an interaction of p53 with basal transcription factor(s) (Seto *et al.* 1992; Mack *et al.* 1993; Ragimov *et al.* 1993; Farmer *et al.* 1996). Transcriptional repression by p53 occurs through interaction with histone deacetylases (HDACs) and co-repressor mSin3a, which binds to the proline-rich region of p53 (Murphy *et al.* 1999; Zilfou *et al.* 2001) and correlates with apoptotic activity. For example, p53 represses the expression of the anti-apoptotic Bcl-2 gene through the TATA sequence in the Bcl-2 P2 minimal promoter (Miyashita *et al.* 1994; Wu *et al.* 2001).

4.1. p53-mediated cell cycle arrest

p53 mediates G_1 arrest through several genes. These include $p21^{WAF1/CIP1}$ (El-Deiry *et al.* 1994), which is the most critical mediator of the p53-mediated G_1 arrest response. The $p21^{WAF1/CIP1}$ protein enables the accumulation of unphosphorylated form of the retinoblastoma (RB) protein by inhibiting several cyclin dependent kinases (CDKs). pRB then arrests cells in G_1 by sequestering the S-phase promoting E2F family of transcription factors (Giaccia and Kastan 1998; Sherr 1998).

p53 may also act in control of a spindle checkpoint or a G_2 checkpoint. p53 mediates G_2/M block after DNA damage (Agarwal *et al.* 1995; Stewart *et al.* 1995) via induction of 14-3-3σ gene (Hermeking *et al.* 1997) preventing premature entry into S phase. It has been hypothesized that p53 interacts with the DNA replication apparatus and directly interferes with DNA replication. One of the evidences for this hypothesis is that p53 physically interacts with and inhibits the function of a cellular DNA replication factor, the single-stranded DNA-binding protein complex RPA (Dutta *et al.* 1993). The interaction between $p21^{WAF1/CIP1}$ and PCNA has also been shown to inhibit the role of PCNA in DNA replication (Li *et al.* 1994; Waga *et al.* 1994). p53 has also been shown to be possible regulator of the homologous recombination through the binding and inhibition of Rad51 (Sturzbecher *et al.* 1996). p53 plays a role in monitoring abnormal recombination intermediates and kills such cells (Guidos *et al.* 1996). p53 ensures the maintenance of diploidy of the cells preventing the large-scale genomic aberrations as observed in the absence of wild-type p53 where the cells reinitiate DNA synthesis that can result in the increase of the ploidity of cells (Cross *et al.* 1995). Cells with wild-type p53 do not undergo gene amplification readily, but p53-deficient cells become tetraploid or octaploid (Livingstone *et al.* 1992; Cross *et al.* 1995). These data explain the genomic instability phenotype that has been described in p53-deficient mice (Donehower *et al.* 1992).

4.2. p53-mediated apoptosis

About a decade after the discovery of p53, many evidences confirm that the ability of p53 to control apoptosis contributes to its tumor suppression activity. In the cells with inactivated p53 the apoptotic response is also hindered, allowing the cells to survive under stress situation. Although there is evidence about the existence of the p53-independent apoptosis, the importance of p53 in regulating programmed cell death is predominant in tumor suppression, because it protects cells from malignant transformation. The role of p53 in apoptosis control has therefore become one of the most extensively studied areas in p53 research, especially in searching for the strategies for restoration of functional

cell death pathways in human tumors. The most promising possibility of restoring the p53-dependent apoptosis include p53-reactivating drugs, small peptides, that recover wild type activities of the mutant p53 protein and could be therefore effective in cancer therapy (Abarzua *et al.* 1996; Selivanova *et al.* 1997; Foster *et al.* 1999; Selivanova *et al.* 1999; Bykov *et al.* 2002).

Stimuli such as severe DNA damage, withdrawal of growth factors, and deregulated expression of oncogenes trigger p53-dependent apoptosis. Both p53-mediated transcriptional activity and the p53 activities not requiring transcription can play a role in apoptosis and the choice depends on the cell type. It is also possible that transcription-regulating functions of p53 co-operate with its transcription-independent functions in the induction of apoptosis. This means that p53 uses transcriptional activation or direct protein signaling (protein-protein interactions or some other activity) to initiate apoptosis. p53 induces expression of several cofactors that have role in apoptosis promotion or inhibition of survival signaling (Figure 3).

Transcription-dependent functions of p53. The process of programmed cell death proceeds through two main pathways: extrinsic and intrinsic, and p53 is involved in both of them. p53 can activate extrinsic apoptotic pathway through the induction of genes encoding transmembrane proteins such as the members of the death receptor family Fas/CD95/APO1 (Owen-Schaub *et al.* 1995), DR5/KILLER (Wu *et al.* 1997), and Pidd (p53-induced protein with a death domain) (Lin *et al.* 2000) that mediate the p53-dependent inhibition of cell growth by inducing apoptosis.

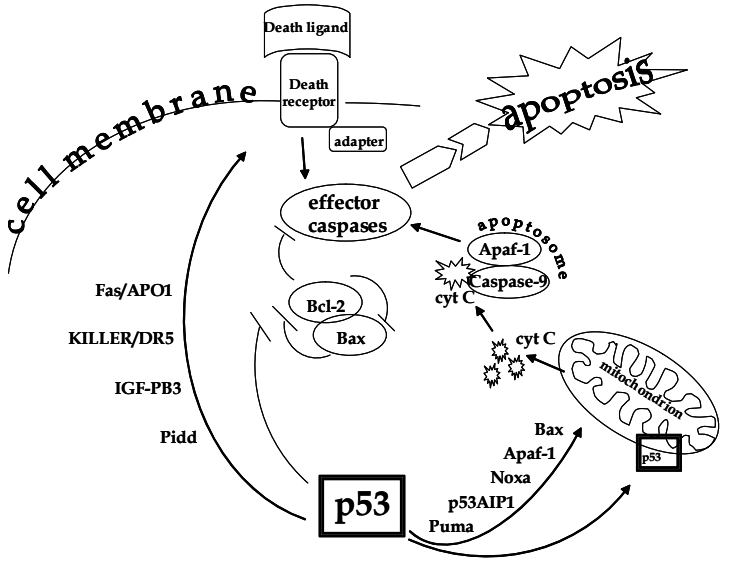

Figure 3. Overview of apoptotic functions of p53.

The intrinsic apoptotic pathway is ruled by the Bcl-2 family of proteins that regulate the release of cytochrome c from the mitochondria (Cory and Adams 2002; Kuwana *et al.* 2002). These include Bax (Selvakumaran *et al.* 1994; Miyashita and Reed 1995), Noxa (Oda *et al.* 2000a), Puma (p53 upregulated modulator of apoptosis) (Nakano and Vousden 2001) and Bid (Sax *et al.* 2002), which are all the transcriptional targets of p53. The pro-apoptotic Bid connects the extrinsic death receptor pathway to the intrinsic pathway, the basis of which underlies in the mitochondrial-disruption processes. p53 modulates the intrinsic and extrinsic pathways through the regulation of Bid (Sax *et al.* 2002). Wild-type p53 phosphorylated at serine 46 induces the expression of p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1). p53AIP1 is localized within mitochondria and mediates p53-dependent apoptosis by disrupting mitochondrial function (Oda *et al.* 2000b).

In addition to the promotion of cytochrome c release through the induction of its target genes, p53 also induces Apaf-1 (apoptosis protease-activating factor 1) expression through a response element within the promoter of the APAF-1 gene (Kannan *et al.* 2001; Moroni *et al.* 2001; Robles *et al.* 2001; Rozenfeld-Granot *et al.* 2002). Activation of caspase proteinases is the central event in the effector phase of apoptosis (Wolf and Green 1999). Caspase-9 can initiate an enzyme cascade that promotes cell death. Released cytochrome c associates with Apaf-1 and caspase-9 to form the apoptosome (Adams and Cory 2002). Caspase-9 with its cofactor Apaf-1 are required for stress-induced p53-dependent apoptosis of fibroblasts and also for Myc-induced apoptosis (Soengas *et al.* 1999; Schuler and Green 2001). Apaf-1 is often found to be inactivated in malignant melanoma where p53 mutations are rare (Soengas *et al.* 2001). Fibroblasts deficient in caspase-9 and Apaf-1 are resistant to p53-induced apoptosis, and are prone to oncogenic transformation by myc (Soengas *et al.* 1999). Apaf-1 may thus substitute for p53 loss in promoting tumor formation since its deficiency is enough to abrogate the apoptotic effect of p53.

The list of the apoptosis-related transcriptional targets of p53 does not end here. Another p53-inducible gene has been identified, termed p53DINP1 (p53-dependent damage-inducible nuclear protein 1) that regulates p53-dependent apoptosis by interacting with a multiprotein kinase complex, which mediates the phosphorylation of p53 at serine 46 (Okamura *et al.* 2001). p53 induces also the expression of Fas ligand (Maecker *et al.* 2000), and IGF-BP3 (Buckbinder *et al.* 1995). The latter sequesters the cell survival factor insuline-like growth factor-1 (IGF-1) so preventing its interaction with its receptor and therefore inhibiting both survival and mitogenic signals from IGF-1. p53 induces the expression of WIP1 (also known as PPM1D) gene in response to genotoxic stress (Fiscella *et al.* 1997). The Wip1 protein is a phosphatase that prevents the phosphorylation of p53 at serines 33 and 46 by p38MAP kinases and activation of the apoptotic function of p53. This suggests for one more potential feedback loop regulating p53 activity (Takekawa *et al.* 2000). The finding that WIP1 gene is amplified in a considerable amount of breast cancers that have retained

wild-type p53, gives additional explanation for the development of human cancers by suppressing p53 activation (Bulavin *et al.* 2002).

Direct role of p53 in mitochondria. p53 also participates in apoptosis induction by acting directly at mitochondria. Stress-induced wild-type p53 protein rapidly translocates to mitochondria where it binds through its DNA-binding domain with Bcl-X_L and Bcl-2 proteins. The interaction of p53 with these proteins prevents them to bind bak and bax and results in cytochrome c release and pro-caspase-3 activation (Mihara *et al.* 2003). Mutant p53 proteins that contain the so called hot-spot mutation in the core domain are defective in their ability to interact with Bcl-X_L and Bcl-2, alluding to the possibility that the selective pressure in human tumors to mutate p53 could be related to this binding (Mihara *et al.* 2003).

GSK-3β has also role in mitochondria during the p53-dependent apoptotic signaling besides promoting p53-mediated transcription by interaction with and by modifying p53 in the nucleus. After DNA damage, mitochondrial GSK-3β interacts with p53 and promotes cytochrome c release and caspase-3 activation (Watcharasit *et al.* 2003). It has been shown that p53 can mediate apoptosis also without its DNA-binding domain (Haupt *et al.* 1995). This could be explained with the GSK-3β, which interacts with p53 through the N- and C-terminus (Watcharasit *et al.* 2003).

Preliminary studies of mutant p53 indicate strongly that loss of the apoptotic activity of p53 is essential for tumor progression. The fact that some tumors retain wild type p53 can be explained with the identification of apoptotic cofactors required for p53-mediated cell death. Because the mutations occur in the p53 downstream components of the apoptotic pathways, they are also considered the targets for inactivation in human cancers (Soengas *et al.* 1999; Eischen *et al.* 2001; Samuels-Lev *et al.* 2001; Soengas *et al.* 2001). Indeed, the inhibition of the expression of p53-inducible apoptotic target genes correlates often with a decrease in p53-induced apoptosis. In some cases, however, this reduction is incomplete. Thus, the p53-dependent apoptosis is a complex process involving numerous p53-inducible apoptotic target genes. The relative contribution of these genes to the full apoptotic response and the role of other functions of p53 that do not depend on activation of gene expression remain to be determined.

4.3. Role of survival signals in p53 functioning

Interplay between p53 and antiapoptotic PI3K-Akt/PKB signaling pathway has a significant role in deciding whether a cell will live or die (Sabbatini and McCormick 1999). Akt/PKB kinase is induced by a variety of growth factors and other survival signals e.g. Ras- and cytokine-mediated signaling pathways that can inhibit p53-induced apoptosis (Kauffmann-Zeh *et al.* 1997).

Akt interacts with Mdm2, phosphorylates it and causes translocation of Mdm2 from the cytoplasm into the nucleus, where it interacts with p300. This, in turn, inhibits the interaction between Mdm2 and p19ARF resulting in increased p53 degradation (Mayo and Donner 2001; Zhou *et al.* 2001). However, interruption of Mdm2-ARF interaction is not the only mechanism for hindering the p53-mediated cell death, as Akt can inhibit apoptosis also downstream of p53 activation, at a later stage in the p53-mediated apoptosis pathway. WISP-1 oncogene (Wnt-1-induced secreted protein), which is induced by the Wnt-1-β-catenin pathway, can activate Akt/PKB signaling pathway leading to inhibition of the mitochondrial release of cytochrome c and up-regulation of antiapoptotic Bcl-X(L). In this case, the enhanced degradation of the p53 protein is not required (Su *et al.* 2002). It has also been observed that growth factors that activate Akt, downregulate the expression of PUMA, which is a mediator of the p53-dependent apoptotic response (Han *et al.* 2001; Nakano and Vousden 2001).

Three mechanisms have been described that counteract PI3K-Akt/PKB-dependent cellular survival, enabling the p53-dependent apoptotic signaling to domineer:

1) One of the negative regulators of Akt-signaling is tumor suppressor PTEN, a lipid phosphatase that dephosphorylates PI3K, thereby impairing Akt activation resulting in inhibiting pro-survival activities (Simpson and Parsons 2001). The expression of PTEN is induced by p53 and has been shown to be essential for p53-mediated apoptosis in immortalized mouse embryonic fibroblasts (Stambolic *et al.* 2001), giving an example how the outcome of the p53 response could be determined. Due to PTEN action, Mdm2 is kept in the cytoplasm and degraded (Mayo *et al.* 2002).
2) p53 is able to induce the down-regulation of Akt protein level through caspase-mediated cleavage (Gottlieb *et al.* 2002). Again, this shows that cell fate depends on the balance between opposite signals of death and survival.
3) p53 induces the expression of cyclin G which in turn recruits the phosphatase PP2AB to the Mdm2-p53 complex, where it dephosphorylates Mdm2 at the Akt phosphotylation sites (Oren *et al.* 2002).

These mechanisms build up a fine balance between p53 and the Akt survival pathway that is often interrupted in human cancers either by loss of PTEN, amplification of Mdm2 or through amplification of PIK3CA, the catalytic subunit of PI3K resulting in enhanced activity of the PI3K-Akt signaling cascade (Mayo and Donner 2002; Singh *et al.* 2002).

5. ARF-p53 pathway

The discovery of ARF in the p16INK4A locus has answered the question how p53 becomes stabilized by viral and cellular oncogenes. The INK4A-ARF gene encodes two distinct tumor-suppressor proteins p16INK4A and p19ARF (or human homologue p14ARF) (Quelle *et al.* 1995). Mutations and the inactivation of this gene are second common events in human cancer after p53. p16INK4a is a negative regulator of cyclin D1-Cdk4 and restrains cell growth through preventing the phosphorylation of the Rb protein. p19ARF acts in ARF–Mdm2–p53 pathway where it prevents the Mdm2-mediated degradation of p53 (Pomerantz *et al.* 1998; Stott *et al.* 1998; Zhang *et al.* 1998b).

p19ARF-deficient mice that express functional p16INK4a develop tumors early in life and primary cells from such mice give continuously proliferating cell line. The introduction of ARF into such cells causes growth arrest, but not in p53-null cells, suggesting, that ARF acts upstream of p53 (Kamijo *et al.* 1997). Disappearance of ARF or p53 function is sufficient to bypass senescence and can immortalize mouse embryonic fibroblasts (MEFs), mouse pre-B cells and keratinocytes. Bone marrow-derived macrophages and astrocytes require loss of both p19ARF and p16INK4a function to grow permanently (Kamijo *et al.* 1997; Holland *et al.* 1998; Lin and Lowe 2001; Randle *et al.* 2001). Most primary human cells regulate senescence through dual mechanism, involving both INK4A-RB and ARF-p53 pathways meaning that INK4A plays a more dominant role in human cells than in the mouse (Brookes *et al.* 2002; Hahn and Weinberg 2002).

The general concept is that ARF connects p53 and oncogene (Figure 4). ARF expression is induced by hyperproliferative signals emanating from dominant oncogenes such as Myc (Zindy *et al.* 1998), E1A (de Stanchina *et al.* 1998), mutated Ras (Lin *et al.* 1998; Palmero *et al.* 1998), from deregulated E2F (Bates *et al.* 1998b; Sherr 2001), and v-Abl (Radfar *et al.* 1998). ARF activation, in turn, by opposing Mdm2 function, leads to the p53-dependent apoptosis or premature senescence depending on the biological context.

E1A and E2F1 activate the transcription of ARF and stabilize p53, but not in the case where ARF is absent (Bates *et al.* 1998a; de Stanchina *et al.* 1998). Activation of E2F1 and myc lead to induction of ARF in part through DAP kinase, which leads to the inhibition of Mdm2 and the stabilization of p53 (Raveh *et al.* 2001).

Ras-driven Raf/MEK/MAP kinase pathway induces Mdm2 gene expression resulting in the degradation of p53, if ARF is absent (Ries *et al.* 2000). Raf also activates the expression of p19ARF, suggesting the mechanism how ARF protects p53 from inactivation by Ras and enables p53-mediated response (Palmero *et al.* 1998). Opposing effects of Ras on p19ARF and Mdm2 therefore can determine the level of the p53 protein. In normal cells, the protective response to mitogenic signals by Ras is dampened by concomitant activation of

Mdm2 and inhibition of ARF by JunD (Weitzman *et al.* 2000). Unlike Myc, expression of high levels of oncogenic Ras in primary MEFs, leads to p53-dependent cell-cycle arrest (Serrano *et al.* 1997). This could account for the transformation of ARF-null MEFs stimulated to proliferate by oncogenic Ras. These cells are resistant to p53-dependent apoptosis induced by DNA-damage although they still contain wild-type p53. Thus, the balance between these two pathways is shifted towards to the cell survival in these cells.

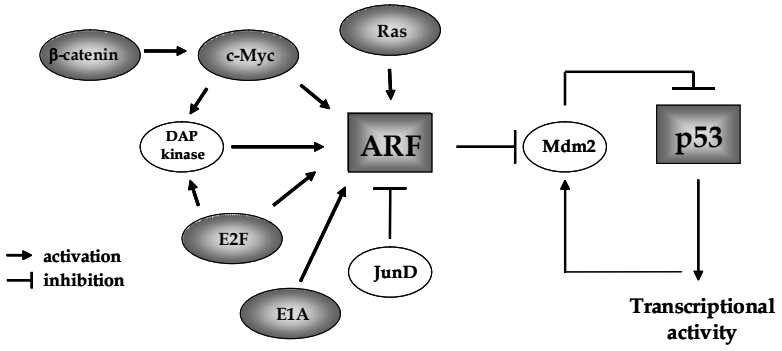

Figure 4. Simplified representation of the ARF-p53 pathway.

Another oncogene that has been shown to activate p53 via ARF is β-catenin (Damalas *et al.* 2001). β-catenin has dual functions being structural component in cell-to-cell adhesion sites and acting in the nucleus as a Wnt signal transducer activating the transcription of target genes with TCF/Lef complex. Moreover, β-catenin can induce the expression of WISP-1 (Wnt-1-induced secreted protein) oncogene which has been shown to activate the antiapoptotic Akt/PKB signaling pathway and therefore inhibit p53-dependent apoptosis, even after DNA damage (Su *et al.* 2002). Deregulated β-catenin can promote tumor development as indicated by the studies of different tumors, especially those of colorectal origin (Roose and Clevers 1999; Peifer and Polakis 2000; Polakis 2000). This happens due to the inactivation of p53 (Kinzler and Vogelstein 1996), ARF (Esteller *et al.* 2000), inactivation of APC (Fodde *et al.* 2001), stabilizing mutations in β-catenin itself or inactivation of other components in growth-suppression pathways. Activated p53 is able to inactivate and induce the degradation of β-catenin through different mechanisms (Liu *et al.* 2001; Matsuzawa and Reed 2001; Sadot *et al.* 2001). β-catenin and p53 thus form a feedback mechanism acting in the opposite directions.

ARF-p53 pathway functions separately from DNA damage response pathway, because ARF-null cells are still able to respond to DNA damage

(Kamijo *et al.* 1997). ARF has been believed to be the central factor in p53-dependent oncogene-induced growth suppression as it is able to protect p53 from Mdm2-mediated degradation. However, recent works have shown that the story of ARF is more complex. Several studies have shown the p53-independent functions of ARF (Lowe and Sherr 2003). For example, mice that lack simultaneously ARF, p53 and Mdm2 develop a broader spectrum of tumors than those lacking p53 alone, or both p53 and Mdm2. Introduction of p19ARF into triple-null MEFs results in cell-cycle arrest in G1 phase, although more slowly than in cells containing Mdm2 and p53. No such effect is seen with MEFs lacking only p53 and ARF (Weber *et al.* 2000). Thus, the ARF-Mdm2-p53 pathway is not strictly linear and in the absence of Mdm2, p19ARF might interact with targets other than p53 to inhibit cell proliferation.

ARF is among the most frequently mutated tumor suppressor loci in human cancer and the data gathered about ARF are as contradictory as about p53. Irrespective of the knowledge about both the p53-dependent and independent ARF functions in the cells, there remains a challenging problem to solve, how cells distinguish between normal and abnormal growth signaling and what is the threshold of such signaling in activating ARF?

RESULTS AND DISCUSSION

1. Aims of the present thesis

Tumors tend to have defects in tumor suppression pathway, through inactivation of p53 either itself or its upstream or downstream mediators. p53 can be inactivated, for example, through the loss of the p53 gene or its coexpression with a dominant negative mutant. The modulation of p53 activity has been actively studied since its discovery.

During the studies on the activity of tumor-suppressor protein p53, specific aspects made up the goals of different projects that gave the content for the present thesis.

The aims of this work were as follows:

- To investigate the ability of mutant p53 proteins to inhibit the activity of wild type p53 protein;

- To examine the influence of electroporation as a commonly used transfection method on the cell cycle and viability;

- To find out whether p53 could respond to CD43 overexpression commonly found in colon tumors, but not in normal colon.

2. Mutant p53 can inhibit the activity of wild type p53 without heterooligomerization (I)

The generally accepted mechanism for the inactivation of wild type p53 by mutant forms of this protein is the formation of mixed tetramers, which are inactive for growth suppression functions. Monomeric p53 protein (that does not oligomerize due to the absence of the required region) should be, therefore, resistant to the dominant negative influence of the mutant proteins. To test this hypothesis, we investigated the influence of tumor associated point mutants (I, Figure 1) to the transcriptional activity of monomeric p53 protein (Δ324-355) in Saos-2 cells. Surprisingly, coexpression with different point mutants (Trp248, His175 and Ala143) clearly inhibited the transactivation function of monomeric p53 (Δ324-355; I, Figures 2a and 3), so did also the point mutant deleted either on N- or C-terminus (ΔN39Trp248 and ΔC362Trp), but not the point mutant deleted from both termini (40-361Trp248; I, Figures 2b and 3). The phenomenon was similar in the case of all reporter constructs used. First of

them contains a synthetic p53 binding site in front of the adenovirus E2 promoter. This binding site, named CON, binds p53 very efficiently and does not occur in natural promoters. Second plasmid contains the 2.4 kb fragment from human WAF1 promoter in front of the CAT gene (El-Deiry *et al.* 1993). The third plasmid has the p53 binding site from Mdm2 promoter. Last two reporter plasmids enabled us to test our finding in a more physiological context. We also carried out the same experiment with a reporter containing the CMV promoter in front of β-galactosidase gene and as shown on Figure 4 (I), the point mutants did not affect the p53-independent transcription.

In order to elucidate the relevance of our results in a more biological assay, we tested the influence of point mutants on the growth suppressive activity of Δ324-355. It has been reported that monomeric form of p53 is also able to suppress growth, albeit to lesser extent than wild type protein (Shaulian *et al.* 1993; Thomas *et al.* 1996). Growth suppression assay showed that wild type p53 inhibited colony formation almost entirely and Δ324-355 reduced the number of colonies approximately 50% as compared to the transfections with the vector carring the resistance marker gene (pBabe Puro) only. Expressing Trp248, ΔN39Trp or ΔC362Trp together with Δ324-355 clearly inhibited the growth suppressive function of the latter (I, Figure 8). The same mutants did not affect notably the colony formation activity, when expressed alone with pBabe Puro (data not shown). We also ascertained by Western Blotting and immuno-fluorescence analysis that the inhibition of the activity of Δ324-355 by point mutants does not occur because of the change in the expression level of the monomeric p53 or the prevention of its nuclear localization when coexpressed (I, Figures 5 and 6). Neither did mutant proteins heterooligomerize with Δ324-355 in described expression conditions (I, Figure 7). Taken together, our data suggest that in addition to heterooligomerization, also other mechanisms can be involved in the inhibition of wild type p53 activity in cells expressing both wild type and mutant p53.

An excess of mutant p53 seems to be required to affect wild type p53 (Sun *et al.* 1993; Park *et al.* 1994; Williams *et al.* 1995; Davis *et al.* 1996). Most tumor-associated mutations in p53 are clustered in the central part of the molecule and they inactivate the DNA binding function of p53. Some of these mutations affect the overall conformation of the central part of the molecule (Gannon *et al.* 1990), whereas conformations of the N- and C-termini are most probably not changed. Therefore, mutant p53 molecules incapable for DNA binding retain the ability to interact with transcription cofactors. If the mutant form of p53 is expressed at a high level, it may deplete cells from cofactors, necessary for p53 to activate transcription, alluding to the competition between wild type and mutant protein for cofactor binding. To explain our results, we propose that mutant p53 interacts with factor(s) specifically needed for p53-dependent transactivation depleting the cells from them, but not for transcription in general, as over-expression of mutant p53 does not alter the expression from the CMV

promoter (I, Figure 4), the activity of which is also dependent on general transcription factors.

The transactivating function of p53 can be modified by different factors that have been discussed in the literature review section of the present work. p53-dependent transactivation and transrepression has been reported to require its interaction with p300/CBP, a coactivator that also interacts with the RelA subunit of nuclear factor-kappaB (Avantaggiati *et al.* 1997; Gerritsen *et al.* 1997; Lill *et al.* 1997; Perkins *et al.* 1997). NF-κB can inhibit p53 function by competing for the co-activator p300 in a dose-dependent manner (Ravi *et al.* 1998; Webster and Perkins 1999). Such a mechanism of coactivator sequestration seems to be common for DNA-binding proteins. Thus, p300 could be one of the cofactors being occupied by the excess of its mutant forms, as p53 is able to form a specific protein complex with p300 both in wild type and in mutant conformation (Avantaggiati *et al.* 1997). Thus, loss of wild type activity can occur through the loss of required co-activators. Another example could be the tumor-suppressor protein p33ING1 that cooperates with p53 and modulates p53-dependent transcriptional activation. The biological effects of these proteins depend on each other; suppression of one of them causes also the growth inhibition (Garkavtsev *et al.* 1998). Several other proteins have been described that interact with p53 and modulate its activity (WT-1, HMG-1, BRCA1, GSK-3β and others). It is not known, whether they can interact with point mutants of p53 or with monomeric p53. Therefore we have no clues to speculate, which one else could be depleted from cells by mutant p53 in our experiments.

Two explanations can be proposed to the fact that Trp248 mutant deleted in both N- and C-terminus cannot inhibit Δ324-355 mediated transactivation. First, the deletions of both ends in 40-361Trp may affect the overall conformation of the molecule making it unable to bind the specific cofactor. This explanation comes from hypothesis according to which N- and C-termini are close to each other in three-dimensional structure. They could generate a binding site for this proposed cofactor and only the deletions of both termini abolish the binding of the cofactor. Second, wild type p53 needs a specific modification both at N- and C-terminus for its activity in addition to binding to a specific cofactor. We suggest that the inhibitory effect of point mutants to Δ324-355 mediated transcription relays on the saturation of the modifying enzymes. Point mutants expressed in higher amount could titrate the Δ324-355 out. The depletion of either N- or C-terminal modifying enzyme is enough to inhibit the transcriptional activity of the monomeric p53. Only in the case of 40-361Trp the Δ324-355 retains its activity, because the mutant deleted in both termini is not able to bind any modifying enzyme. This also implies for several cofactors that cooperatively modulate the p53 activity through protein-protein interaction and/or modification of p53, because the deletion of either N- and C-terminus alone was not sufficient to abolish the dominant-negative effect of the mutant.

Last, in our hand p53 mutants inhibited the growth suppression by monomeric p53. p53 suppresses growth both through its transcativation activity and the trans-repression of promoters without classical p53 binding sites, which does not need DNA-binding function, but still make use of chaperon proteins. If the modifying enzymes and cofactors targeted to the termini of wild type molecules are also able to bind to mutant p53, mutant p53 can compete with wild type p53 itself. This theory is partly supported by a recent work where it has been shown that mutant p53 inhibits the ability of wild-type p53 to bind to the p53 responsive element in the promoter of its target genes and subsequently its ability to transactivate its target genes (Willis *et al.* 2004).

In conclusion, the dominant negative effect of mutant p53 proteins over wild type p53 can act through the combination of different mechanisms in addition to the forming of heterooligomers, including the competition for the p53-specific cofactors and the inhibition of DNA binding of wild type p53.

3. Electroporation causes p53 activation, cell cycle arrest, and apoptosis (II)

In research works, the cellular effects often need to be studied using ectopic expression of DNA sequences. Several methods have been developed for introducing foreign DNA, carrying a gene of interest, into the cell. Electroporation is one of the widely used methods. This procedure is based on the exposure of cells mixed with DNA into external electric field, which brings cell membrane to the permeabilized state that enables the plasmid DNA to enter into the cells (Golzio *et al.* 1998).

In response to various genotoxic agents, p53 protein accumulates in the nuclei of mammalian cells leading to either p53-dependent growth arrest or apoptosis. Electroporation procedure can cause cellular stress as it may change the balance of ions inside the cell, activate cellular nucleases, or damage cellular DNA directly. We were therefore interested in the potential effect of electroporation on the cells, especially, whether it has any influence on p53-dependent response pathways to cellular stress.

To investigate this idea we used mouse cell lines NIH3T3 and 10(1), which are p53 positive and negative, respectively. Since this transfection method also involves the addition of carrier DNA, we electroporated the cells in the absence and presence of carrier DNA (sonicated yeast DNA) followed by BrdU labeling 24 h posttransfection and the determination of percentage of cells in S phase using flow cytometry. Indeed, the electroporation procedure itself induced S-phase arrest in both cell lines. This effect was more pronounced when carrier DNA was present during electrical pulse (II, Figure 1). The cells also underwent apoptosis under the same conditions and the dying of cells considerably

increased by the addition of carrier DNA (II, Figure 2). These results indicate that electroporation procedure causes cellular stress resulting in the death of considerable amount of cells. The activation of p53-independent pathways seems to be sufficient under these circumstances, because the cells responded similarly in p53-positive and p53-negative background.

Next, we were interested in the potential involvement of p53 protein in response to electroporation induced cell stress. To investigate this idea, we studied the effect of electroporation and carrier DNA on the levels and transcriptional activity of endogenous p53 protein. We examined electroporated NIH3T3 and MCF-7 cells both containing wild type p53. Western blot analysis with antibodies specific to p53 and its target gene products Mdm2 and p21 revealed that the electroporation procedure caused elevation in the levels of p53 protein and the upregulation of its target genes Mdm2 and p21 compared to nontransfected cells (II, Figures 3 and 4a, lanes 2 and 1, respectively). Carrier DNA electroporated into the cells induced additional increases in the levels of these proteins (II, Figure 3 and 4a, lane 3). Transfection of supercoiled plasmid DNA had similar influence on the level of p53 as carrier DNA (II, Figure 4a, lane 4). p53 levels were not additionally elevated also in the case of transfection of non-relevant expression plasmid either in the absence or presence of carrier DNA (II, Figure 4a, lanes 5 and 6). Based on these observations, we assume that endogenous p53 is stabilized by the electroporation procedure. Addition of foreign DNA causes a further rise in p53 levels irrespective of the concentration or nature of added DNA. Consequently, electroporation seems to be unsuitable method for introducing a gene of interest into cells, if the cell behavior in response to stress stimuli is under investigation.

We have looked for an alternative transfection method to avoid the negative side effects of electroporation. Polyethylenimine (PEI) is a cationic polymer that is an effective carrier for delivering genes into the mammalian cell. It complexes with DNA, condenses DNA into small particles and enables DNA to reach into the nucleus (Boussif *et al.* 1995; Mislick and Baldeschwieler 1996; Demeneix *et al.* 1998).

We tested the effect of PEI-mediated transfection on the growth and survival of cells, using BrdU incorporation assay and annexinV staining, respectively. In both NIH3T3 and 10(1) cells neither PEI itself nor PEI-transfected pcDNA caused any reduction in the percentage of S-phase cells (II, Figure 5). Carrier DNA triggered a significant S-phase block in 10(1) cells and almost completely arrested cell division in NIH3T3 cells, but in the case of linearized pcDNA we observed no considerable change in the number of S-phase cells compared to the non-treated cells (II, Figure 5). Considerable part of the carrier DNA-transfected NIH3T3 cells underwent apoptosis during 48 h posttransfection, at the same time in 10(1) there was only a slight rise in the number of apoptotic cells. Linear pcDNA gave an intermediate effect: in NIH3T3 it triggered 3–4 times more apoptotic cells than supercoiled pcDNA but two times less than carrier DNA (II, Figure 6). NIH3T3 cells died considerably with PEI only, but

not when PEI was complexed with DNA. Observation that NIH3T3 cells transfected with carrier DNA and linear pcDNA responded with reduction in S-phase and increase in apoptosis could be explained by the presence of endogenous p53.

To test this idea, we studied the level and transcriptional activity of endogenous p53 in NIH3T3 and MCF7 cells after PEI-mediated transfection. In both cell lines neither PEI nor PEI-transfected pcDNA affected the levels of p53 or its transcriptional target Mdm2 (II, Figures 7a and 7b, lanes 2 and 3). In MCF-7 cells we also examined the overexpression of Δ1-125CD43 that did not affect p53 or Mdm2 levels (II, Figure 7b, lane 5), but p14 ARF elevated both the levels of p53 and Mdm2 as expected (II, Figure 7b, lane 4).

Taken together, our results show that electroporation causes cellular stress resulting in cell cycle arrest and apoptosis both in p53-positive NIH3T3 and p53-negative 10(1) cells. PEI-transfection on the other hand did not cause such severe stress response in these cells. In the case of electroporation, this procedure may damage cellular DNA or activate cellular nucleases by changing the balance of ions inside the cell. Carrier DNA delivered into the cells can mimick DNA damage and augments the effect of this transfection method. This could lead to activation of p53-dependent and p53-independent cell-cycle control mechanisms, thereby altering the outcome of the experiment. Efficiency of PEI-mediated transfection relies on the lysosome buffering capacity of the DNA/PEI complex that protects DNA from nuclease degradation and facilitates the release of the PEI/DNA complex (Boussif *et al.* 1995). PEI-mediated transfection thereby seems to be a better method for cell biological studies, because it causes no side effects.

4. Studies on the modulation of the activity of p53 by CD43

4.1. CD43 in tumorigenesis – short introduction

CD43 is a typical member of the cell surface mucins, molecules that play a relevant role in tumor progression. CD43 is a transmembrane sialylated glycoprotein expressed on the surface of most hematopoietic cells (Andersson and Gahmberg 1978; Remold-O'Donnell *et al.* 1984; Carlsson and Fukuda 1986). The precise function of CD43 is unclear. CD43 appears to be important for the function of the immune system and has been shown to be involved in cell adhesion and cell proliferation. The extracellular domain of CD43 has both adhesive and antiadhesive properties (Ardman *et al.* 1992; Ostberg *et al.* 1996). This is followed by the transmembrane region and evolutionary highly

conserved C-terminal intracellular region that mediates signal transduction (Pallant *et al.* 1989; Shelley *et al.* 1989).

CD43 has long been considered to be expressed exclusively in hematopoietic cells and therefore CD43 has not been studied in other cell types. However, in 1995, CD43 was first found in a colon cancer cell line (Baeckstrom *et al.* 1995) and later the expression of CD43 was reported to be frequent in colon adenomas and carcinomas, where it occurs in the early stages of tumor development. Normal colon epithelial cells do not express detectable levels of CD43 (Sikut *et al.* 1997). Later, another group observed the same phenomen (Pimenidou *et al.* 2004). CD43 expression is also frequent in established human cell lines of different origins (Fernandez-Rodriguez *et al.* 2002). The high frequency of CD43 expression already in the early stages of tumor (adenoma) could suggest an important role of this protein in tumor development (Sikut *et al.* 1997). Moreover, in colon adenoma and carcinoma tissues, CD43 seems to have a more intracellular localization, in contrast to the membrane localization typical for leukocyte-type cells (Sikut *et al.* 1999). One reason for CD43 being unnoticed outside of the hematopoietic lineage is that CD43 is differently glycosylated in other cells, as for example, in the human colon adenocarcinoma cell line COLO205, but the antibodies used so far were developed against the glycosylation-dependent epitopes found on leukocyte-CD43 molecule (Baeckstrom *et al.* 1991).

4.2. Overexpression of CD43 causes activation of the tumor suppressor protein p53

The accumulation of the p53 protein has been shown in early stages of different tumors including colon adenomas, but p53 mutations occur relatively late during colorectal carcinogenesis (Fearon and Vogelstein 1990; Mazars *et al.* 1991). As the expression of CD43 in colon adenomas has been suggested to be also an early event (Sikut *et al.* 1997), it was considered of interest to determine if there was a crosstalk between the presence of CD43 and the p53-response pathway in early tumor development.

In this study, we show that the overexpression of CD43 induces the accumulation of the p53 protein (III, Figure 1). This provides the first evidence that CD43 can trigger the activation of the p53 protein. We investigated this phenomenon by analyzing the level of p53 mRNA. We found that p53 mRNA levels remained the same under conditions of CD43 overexpression (III, Figure 2). We can conclude that the effect of CD43 on p53 is not at the level of mRNA transcription, but is likely to be translational or posttranslational. Examination of the p53 turnover by the pulse-chase method showed that the half-life of p53 is significantly longer after cotransfection with CD43 (III, Figures 3a and 3b). Further studies showed that p53 induced by CD43 is able to activate

transcription from both artificial p53-specific promoter constructs (III, Figure 4) and cellular response genes (III, Figure 5). Namely, Mdm2 and p21, two of the most important p53 response genes, were upregulated under the conditions of CD43 overexpression. These results support the idea that in addition to the activation of p53, CD43 triggers the p53-dependent cellular response to possible mitogenic stimuli. Note, that during this study we observed that electroporation method for introducing foreign DNA into the cells could cause additional stress to these cells (paper II). We therefore used PEI-transfecton method in further experiments.

Post-translational modifications, including phosphorylations, play a role both in stabilizing p53 protein and in activation of its transactivation ability. With the stimulation of CD43 in addition to the increase in the total p53 protein level, there was also a clear increase in the phosphorylation of p53 at serine 15 (III, Figure 3c), showing that accumulated p53 is phosphorylated at this site. Phosphorylation at serine 15 has been reported to impair the ability of Mdm2 to bind p53 (Meek 1998) suggesting that in our case, p53 is stabilized and activated through the disruption of binding with Mdm2. As several kinases can induce p53 serine 15 phosphorylation, there could be more than one upstream regulators of p53 to mediate signaling in response to CD43 overexpression. Among other kinases Erk, a member of MAPK family, has been shown to mediate the phosphorylation of p53 at Ser15, which in turn activates the transactivation function of the p53 protein (Persons *et al.* 2000; Wang and Shi 2001). CD43 has been reported to induce cell proliferation through the activation of Syk-dependent Erk signaling (Miura *et al.* 2001), suggesting that Erk could be one of the possibilities to link CD43-dependent signal transduction pathway to p53. We have not investigated yet, how else p53 could be modified under these conditions.

4.3. The activation of p53 by CD43 is mediated by tumor suppressor protein ARF

An important factor in keeping cell growth under control is ARF protein, which is induced in response to the deregulated oncogenes (Sherr and Weber 2000). Since in our experiments CD43 expression led to an increase in the level of endogenous ARF protein in 10(1) cells (III, Figure 6a), it is clear that in this case p53 activation operates via ARF tumor suppressor protein. The main role of ARF activation is the stabilization of p53 by Mdm2 inhibition and activation of p53-dependent growth suppression. If cells lack ARF, they fail to inhibit oncogene-induced hyperproliferation. Reintroduction of ARF into these cells restores their ability to respond to oncogenic stimuli (Zindy *et al.* 1998). CD43 was unable to induce p53 in cells lacking endogenous ARF, such as NIH3T3, MCF-7, and LoVo (data not shown). The coexpression of CD43 and ARF

proteins in ARF-null mouse embryo fibroblasts and human MCF-7 cells resulted in the elevation of the endogenous p53 protein level (III, Figures 6b and 6c).

Having determined a link between p53-dependent signaling and CD43, we were interested in the biological outcome of CD43 overexpression in our experimental system after the activation of the p53 protein. Several groups have reported that p53 triggers apoptosis in response to oncogenic overexpression depending on ARF protein, which is important for tumor suppression (Lowe and Sherr 2003). We therefore studied whether the overexpression of CD43 could lead to an apoptotic response. Indeed, CD43 induced apoptosis, if coexpressed with ARF in ARF-null MEFs (III, Figure 7) and with p53 in p53-null 10(1) cells (data not shown), affirming that both p53 and ARF are required for the induction of apoptosis in response to CD43 expression. Taken together, CD43 overexpression leads to the activation of tumorsuppressor proteins ARF and p53 that results in cell death, which is the main mechanism of defense against malignant growth. We propose an idea according to which aberrant CD43 expression in colon epithelial cells could represent an oncogenic stimulus leading to the uncontrolled cell division and resulting in tumor development if the ARF-p53 tumor suppressor pathway is disrupted. The idea is supported by further experiments according to which CD43 also suppressed drastically colony-forming ability of ARF-null MEFs and 10(1) cells only if both p53 and ARF proteins were present compared to the expression of CD43 alone (data not shown). Moreover, in the case where either p53 or ARF was missing, CD43 expression increased considerably the colony forming ability of the cells compared to the control cells (data not shown).

Different molecular events contribute to the development and progression of carcinomas. What kind of mechanism could be responsible for tumor development in the case of CD43? An important role in the regulation of gene expression and cell function has been attributed to CD43-mediated signals. A serine/threonine kinase has been cloned that can specifically interact with the cytoplasmic domain of CD43. This kinase, named Sialophorin tail-associated nuclear kinase (STANK), localizes both in cytoplasm and in the nucleus. It has been suggested that CD43 may mediate its biologic effects through the activation of a kinase cascade, resulting in the expression of specific genes and cell growth (Wang *et al.* 2000). STANK is nearly identical to HIPK2 (Kim *et al.* 1998). HIPK2 has been shown to modulate p53-dependent apoptosis by phosphorylating p53 at serine 46 (D'Orazi *et al.* 2002). As mentioned above, adhesion via CD43 induces tyrosine kinase Syk activation (Miura *et al.* 2001). Syk activity in turn is a key regulator of Akt kinase activity and is also shown to induce downstream activation of ERK and JNK kinases (Stewart and Pietenpol 2001). All these molecules are known to be involved in signal transduction processes in the cell and are therefore attractive candidates linking CD43-mediated signaling to the p53-response pathway (III, Figure 8). Very recently, it has been shown that the cytoplasmic domain of CD43 can translocate into the

nucleus and interacts with β-catenin, an oncogene that acts in cell survival pathways and its overexpression have been shown to activate p53 (Damalas *et al.* 1999; Andersson *et al.* 2004). This finding also supports the idea of the potential role for CD43 in tumor development.

In summary, CD43 overexpression can lead to the activation of cell survival pathways that stimulate cell growth and the activation of p53 in response to these proliferative abnormalities provides a strong protection against malignant growth bringing cells to die. We have established a relation between CD43 and p53 pathways. The exact mechanism of CD43 in triggering p53 response remains to be elucidated and will be of interest for further studies.

CONCLUSIONS

The results of this study can be summarized as follows:

1. p53 protein with tumor-associated point mutations can inhibit the growth suppressing function of wild type p53 without hetero-oligomerization. This ability of the mutant protein requires the presence of either native C- or N-terminus in the mutant protein. Deletion of both 39 amino acids from N-terminus and 32 amino acids from C-terminus abrogated this dominant negative effect, as the mutant having both termini deleted failed to inhibit the activities of the monomeric p53 protein.

   The obtained data suggest that the mutant proteins, when overexpressed, can deplete the cellular pool of p53-specific transcriptional coactivator(s) needed for p53 to activate the p53-specific promoters and mediate its biological effects.
2. Electroporation, despite its wide application for gene transfer into the cells, causes at least in part p53-dependent inhibition of cell-cycle and apoptosis induction. Cell transfection procedure using polyethylenimine did not cause any changes in the levels of p53 and cell cycle. Due to lack of side effects, this method is more suitable for cell biological studies, compared to electroporation.
3. Overexpression of CD43 causes accumulation of p53 in 10(1), ARF-null mouse embryonic fibroblasts and MCF-7 cell lines. The p53 protein induced by CD43 is transcriptionally active, stimulating transcription from p53-responsive promoters.

   CD43 does not induce p53 in the cells lacking ARF, but ARF expression in these cells restores the ability of CD43 to induce p53, resulting in cell suicide. These results lets us to draw the conclusion that the ARF-p53 response pathway could be activated by the possible mitogenic stimuli caused by CD43 expression.

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SUMMARY IN ESTONIAN

Kasvaja supressorvalgu p53 aktiivsuse moduleerimisest

Vähkkasvaja korral on osa rakke organismis kaotanud kontrolli jagunemise üle. Piiramata jagunemise käigus kuhjuvad kasvajarakkudesse geneetilised muutused, mille tulemuseks on tuumorsupressor-geenide inaktiveerumine ja rakkude jagunemist soodustavate geenide kontrollimatu ekspressioon, mida peetaksegi kasvaja tekke peapõhjusteks. Kasvajaseoseliste geenide identifitseerimine ning nende funktsiooni uurimine on aidanud mõista rakkude jagunemise mehhanisme ning annab võimaluse välja töötada vähivastaseid ravimeetmeid.

Üks väga olulisi tegureid rakkude jagunemise kontrollis on tuumorsupressor-geen P53, mis on muteerunud enam kui pooltes uuritud kasvajatest. Käesolevas töös uuritakse selle geeni poolt kodeeritavat valku p53. Raku sattumisel stressi tingimustesse p53 valk peatab rakutsükli ja tõsisema rakusisese kahjustuse korral indutseerib rakkude programmeeritud surma. Oma võime tõttu hoida ära kasvajateket on p53 tabavalt nimetatud ka ’genoomi valvuriks’.

Töö peamiseks eesmärgiks oli uurida erinevaid p53 aktiivsust mõjutavaid mehhanisme, nii aktiveerivaid kui inhibeerivaid. Täpsemad uurimisteemad, saadud tulemused ning nendest tehtavad järeldused on järgmised:

- Normaalne p53 funktsioneerib kõige efektiivsemalt tetrameerina. Erinevates töödes on täheldatud, et kui avaldada rakkudes üheaegselt kasvajaseoselist p53 mutanti koos normaalse p53 valguga, on tulemuseks heterotetrameer, mis ei ole võimeline täitma normaalse p53 funktsioone. Sellest tulenevalt võiks arvata, et monomeerne p53, millel puudub võime moodustada oligomeere, kuid omab endiselt normaalse p53 aktiivsusi, ei ole selliselt mutantide poolt mõjutatav. Üllatuslikult selgus, et mutandid olid võimelised p53 aktiivsust inhibeerima ka ilma heterotetrameere moodustamata. Mutantse p53 inhibeeriv mõju sõltub natiivse N-või C-terminuse olemasolust, sest mõlema otsa deleteerimine kaotas mutantse p53 võime inhibeerida monomeerset p53. Saadud tulemust võib seletada sellega, et p53 mutandid ülehulgas avaldatuna seovad enda külge p53 aktiivsuseks vajalikud kofaktorid, mida on rakus piiratud koguses.
- Elektroporatsiooni meetod, mida kasutatakse uuritava geeni viimiseks rakku, põhjustab rakutsükli peatamise ja rakkude märkimisväärse suremise nii p53-sõltuvalt kui ka sõltumatult. Seevastu rakkude transfekteerimine polüetüleenimiini (PEI) abil selliseid kõrvalefekte ei põhjustanud. Seega on PEI-meetod palju sobivam rakubioloogilisteks uuringuteks.
- Kasvajarakkudes on täheldatud erinevate geenide ebanormaalset ekspressiooni ning üks nendest on vererakkude-spetsiifiliseks peetud CD43. CD43 ekspressioon on tavaline nähtus soolekasvajatekke varajases staadiumis,

samuti p53 valgu taseme tõus. Uurides võimalikku seost p53 taseme tõusu ja CD43 üleekspressiooni vahel leidsime, et CD43 indutseerib p53 aktivatsiooni ja tema märklaudgeenide ekspressiooni. Rakkudes, kus puudus tuumorsupressorvalk ARF, CD43 p53 valku ei indutseerinud. ARF-i ekspresseerimine nendes rakkudes nö. taastas CD43 võime aktiveerida p53, mille tulemusena indutseeriti rakkude surm. CD43 inhibeeris samuti rakkude kolooniate formeerumise võimet nendes rakkudes, mis ekspresseerisid nii ARF- kui p53 valku. Juhul, kui üks nendest valkudest puudus, CD43 märgatavalt suurendas rakkude kolooniate formeerumise võimet võrreldes kontrollrakkudega. Nende tulemuste põhjal võib järeldada, et CD43 soodustab rakkude jagunemist ning kutsub sellega esile p53-ARF-sõltuva signaaliraja aktiveerimise, mille tulemusena indutseeritakse rakkude suremine kui peamine viis kasvajatekke mahasurumiseks.

Kokkuvõttes esitavad käesoleva töö tulemused uuemaid aspekte seoses mutantse p53 valgu võimega inaktiveerida normaalset p53 valku kasvajates nende koosesinemise korral. Samuti esitame versiooni CD43 ekspressiooni kohta mitteverepäritolu kasvajates. Uuringute käigus selgus samuti, kui oluline on rakkude töötlemiseks kasutatava meetodi valik, eriti kui kõne all on raku vastuse uurimine stressi tekitavatele tingimustele.

ACKNOWLEDGEMENTS

The study was carried out at the Department of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu during the years 1996–2004.

The work was supported by grants from the European Commission (CT94002 and QLG7-CT-1999-00851), Estonian Science Foundation (No. 2315, 2316, 4029 and 4569), Swedish Institute, and Swedish Cancer Foundation.

I express my sincere gratitude to my supervisor Professor Toivo Maimets for his support and advice during my research, and for encouraging me during sluggish periods.

I would like to thank my coauthors: Many thanks to Arnold Kristjuhan and Arvi Jõers (you are the best tutor!) for fruitful discussions. Sirle Laos, my 'sunbeam', thank you for lending me your helping hand at the moment I needed it the most, and for your endless optimism and encouragement, always accompanied by your wonderful sense of humor. Thank you, Dina Lepik, for your collaboration, helpful discussions and comments concerning to my thesis. Viljar Jaks, thank you for always having time for me, helping and supporting me in solving the every-day problems occurring in the lab and for the nice chats about life itself, and for valuable suggestions concerning my thesis. And Signe Värv, you are the nicest personality I have ever met.

Many, many thanks to my present and former colleagues for friendly atmosphere and help: Alar Aints, Jaan-Olle Andersoo, Maia-Liisa Anton, Sulev Ingerpuu, Jaak Jänes, Ade Kallas, Johanna Kase, Sirje Kivi, Sulev Kuuse, Triin Mölter, Siim Pauklin, Margus Pooga, Tanel Punga (always encouraging me during my first years in lab), Ilvi Rimm (for the excellent technical help), Dorel Sabre, Anu Sikut (for a nice cooperation), Rein Sikut (for helpful discussions and useful comments concerning my thesis), Tiit Talpsepp and Tiia Tooming.

My thanks belong also to my students: Marja Danil, Kersti Jääger and Marko Lõoke — in addition to being nice young colleagues, by tutoring you, I could learn a lot myself, too.

Thank you, Tõnis Örd and Reet Kurg, for finding time to read my thesis. I am also very thankful to Anne Kalling and Annely Kukk, and to all the other friendly people from the Institute of Molecular and Cell Biology and Estonian Biocentre.

Tiiu Rootslane, thank you for helping me to cope with the bureaucracy throughout all these years and always reminding me if I forgot something.

I am grateful to Professor Gunnar Hansson from Göteborg University for critical revision of the manuscript of the last paper of this thesis, and Christian Andersson for a nice cooperation.

It was a pleasure to study in the group of Professor Matthias Löhr from Rostock University. I warmly thank him and all of the nice people from his group, especially Ralf Jesnowski.

Most of all I wish to express my deepest gratitude to those I love the most:

I am thankful to my mother, the person who directed me towards the scientific world. My father, being always supportive, whenever I needed it; and my two fantastic brothers, Meelis and Elvis, I am very proud of. Dear Magnus, thank you for your love, understanding, patience, and support during all these seven years we have shared together and I promise to have more time for you in the future.

There are many more people to whom I am also very grafeful, who have more or less made an invaluable contribution to facilitate progress in my academic life, and in my evolution from a shy student to the person I am today.

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Since 1995 I have been studying the activity of tumor suppressor protein p53. I have been working at projects dealing with the p53 at the protein level and with the relationship with other proteins concerning oncogenic transformation of cells. This thesis is based on the studies of the modulation of the activity of p53.

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Teadustegevus

Peamiseks uurimisteemaks on olnud alates 1995. aastast kasvajasupressor-valk p53. Olen osalenud projektides, kus uuritakse p53 funktsiooni valgu tasemel ning seost rakkude transformatsiooni põhjustavate valkudega. Käesolev doktoritöö keskendub uurimistööle, mis käsitleb p53 aktiivsust moduleerivaid faktoreid.

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