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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers which will be referred to by their Roman numerals:

I **Kristjuhan A.** and Maimets T. (1995). Protein p53 modulates transcription from a promoter containing its binding site in a concentration-dependent manner. *Eur. J. Biochem.* **234**: 827–831.

II **Kristjuhan A.**, Jaks V., Rimm I., Tooming T. and Maimets T. (1998). Oligomerization of p53 is necessary to inhibit its transcriptional transactivation property at high protein concentration. *Oncogene* **16**: 2413–2418.

III Jõers A., **Kristjuhan A.**, Kadaja L. and Maimets T. (1998). Tumour associated mutants of p53 can inhibit transcriptional activity of p53 without heterooligomerization. *Oncogene*, in press.

LIST OF ABBREVIATIONS

C-terminus	Carboxy-terminus
CAK	CDK-activating kinase
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
Cdc	cell division cycle genes
CDK	cyclin dependent kinase
CKII	casein kinase II
CON	p53 binding consensus DNA sequence
CMV	cytomegalovirus
DNA-PK	DNA-dependent protein kinase
GADD	growth arrest and DNA damage
GAL4	yeast transcription factor
HIV	human immunodeficiency virus
HMG-1	high mobility group protein 1
HPV	human papillomavirus
HSV	herpes simplex virus
HTLV	human T-cell lymphotropic virus
IE	immediate early
IGF	insulin-like growth factor
IGF-BP3	insulin-like growth factor-binding protein 3
JNK	c-Jun N-terminal kinase
LCR	long control region
LTR	long terminal repeat
MAP	mitogen-activated protein
MKC	muscle creatine kinase
N-terminus	Amino-terminus
NER	nucleotide excision repair
PCNA	proliferating cell nuclear antigen
PKC	protein kinase C
Pol	polymerase
Pu	purine
Py	pyrimidine
RGC	ribosome gene cluster
RSV	Rous sarcoma virus
SH3	Src-homology domain 3
ssDNA	single-stranded DNA
SV40	simian virus 40
TAF	TBP-associated factor
TBP	TATA-binding protein
TFIID	transcription factor II D

TFIIH	transcription factor II H
UV	ultraviolet
wt	wild type
WT1	Wilms' tumor protein 1

1. INTRODUCTION

Genome contains a huge set of genes, which all do something and do it at the right time in the right place. They all depend on each other and their activities are often regulated by each other. They form a large network, all components of which exist in the balance. But sometimes something goes wrong and some components of the machinery are not controlled any more. Therefore, cells have developed various systems for repair of different abnormalities. One possible output of uncontrolled life of cells is development of cancer. To prevent this fate, cells have tumor suppressor genes, primary role of which is to protect them from abnormal proliferation.

Tumor suppressor p53 is a very prominent protein: it does almost everything and nothing at the same time. It is involved in regulation of cell cycle and apoptosis, activates and represses transcription, is involved in DNA replication and repair, binds DNA and sometimes degrades it, represses translation of some mRNAs, reanneals single-stranded nucleic acids and has a protease activity, but in spite for all that, it is not necessary for normal life of cells. The only real function of p53 seems to be tumor suppressing, which is needed for prevention of cancer development. Due to its unique role, p53 is very attractive target in cancer therapy and much effort has been made for restoration its activity in cancer cells. Its crucial role in cancer development and numerous activities have also made p53 one of the most intensively studied proteins during past years.

In August 1998, the Medline database contained over 6700 records, which had a word "p53" in the Title of the document. The experimental part of this thesis is based on three of them, which all discuss activation of transcription by p53. The first two papers describe ability of p53 to inhibit its own trans-activating activity at high protein concentrations and the structural requirements for that. Results of the third paper suggest that p53 needs an essential cofactor to be fully active transactivator.

2. REVIEW OF LITERATURE

2.1. p53 protein

2.1.1. p53 and tumorigenesis

After its discovery in 1979 (Lane and Crawford, 1979; Linzer and Levine, 1979) p53 has been one of the most extensively studied proteins. The early observations that p53 is frequently overexpressed in tumor cells, regardless of the transforming agent and the cell type, were the basis for the concept that this protein can serve as a general marker for tumor cells (Rotter, 1983). Later investigations revealed that actually a mutated p53 is overexpressed in tumors, while wt protein is expressed in normal cells at very low level. Further studies indicated that wt p53 has a growth suppressing property and expression of wt p53 prevents cell transformation with many viral and cellular oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Baker *et al.*, 1990). Therefore, p53 was defined as a tumor suppressor gene.

The idea, that p53s primary role is to act as tumor suppressor, was further supported by establishment of p53 knockout mice. The initial result of analysis of $p53^{-/-}$ mice was that they develop normally, but are highly susceptible to spontaneous formation of tumors (Donehower *et al.*, 1992). Later studies, however, indicated that 8–16% of the p53-deficient embryos developed abnormally. In some cases, defects in neural tube development, such as exencephaly, were observed (Sah *et al.*, 1995). In agreement with the notion that p53 is a tumor suppressor gene, $p53^{-/-}$ mice developed a high frequency of tumors, mainly lymphomas, at an early age. However, the particular genetic background of the mice affects tumor incidence and spectrum. Mice heterozygous for inactivated p53 allele also show increased incidence of spontaneous malignancies as compared to $p53^{+/+}$ mice. In this case, the heterozygous mice develop predominately osteosarcomas and soft tissue sarcomas. In the majority of cases with heterozygotes, the wt p53 allele has been lost in tumors (Donehower *et al.*, 1992; Harvey *et al.*, 1993; Jacks *et al.*, 1994; Purdie *et al.*, 1994).

It is now well anticipated that the inactivation of wild type p53 is a key event in tumorigenesis. The best studied way of p53 inactivation is by mutating its gene. Indeed, p53 mutations are found in about 50–55% of all human cancers (Hollstein *et al.*, 1994). Approximately half of the major forms of cancer contain p53 missense mutations, about 40% of which localize to certain "hot spots" along the p53 molecule (Fig. 1). Humans, who are heterozygous for the wild-type allele of p53, develop cancer with a very high frequency (greater than 90–95%) and often at early age.

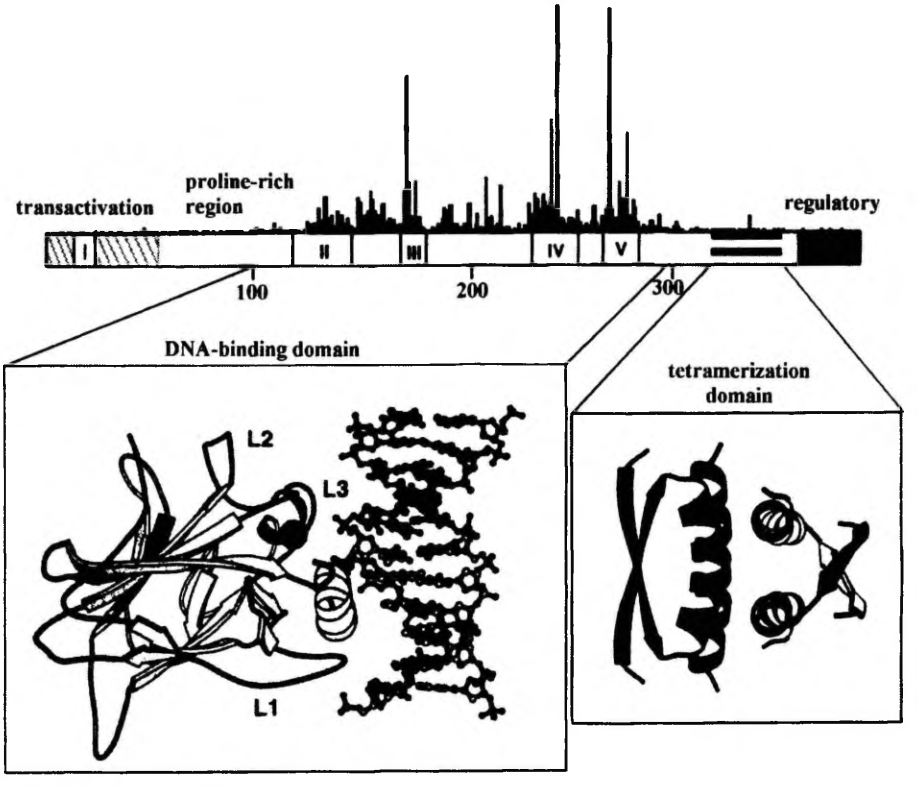

Figure 1. Structure of p53 protein and location of different functional domains. The three-dimensional structures of the DNA-binding domain complexed with DNA (Cho *et al.*, 1994) and tetramerization domain (Lee *et al.*, 1994) are shown as ribbon diagrams of the backbone of the protein. β-strands are shown as arrows and α-helices as coils. The five conserved regions of the protein are indicated in roman numerals. The frequency of tumor-derived mutations at each residue (Cariello *et al.*, 1994) is plotted as histogram along the top.

Less studied, but probably not less important than mutations in p53 gene is the functional inactivation of p53. Some tumors, containing wt p53, have amplified mdm2 gene, product of which leads to inactivation of transactivation activity of p53 and also causes rapid degradation of p53 (Oliner *et al.*, 1992; Reifenberger *et al.*, 1993). The second known mechanism leading to inactivation of wt p53 is its sequestration into the cytoplasm, which was first described in breast cancer cells and subsequently in undifferentiated neuroblastomas (Moll *et al.*, 1995). If we add other, yet unidentified, ways of inactivation of p53 (or its pathway), it is very likely that disruption of p53 activity is necessary for developing most of the tumors.

2.1.2. Structure of p53 protein

2.1.2.1. Functional domains

Human p53 protein contains 393 amino acid residues which can be divided at least into five functional domains (Fig. 1): the transactivation domain (residues 1–40), the "proline-rich" domain (residues 60–90), the DNA-binding domain (residues 100–300), the oligomerization domain (residues 326–355) and the regulatory domain (residues 360–393).

The transactivation domain of p53 was first described in 1990 as a region of p53 which can activate transcription of reporter gene when fused with GAL4 DNA binding domain (Fields and Jang, 1990; Raycroft *et al.*, 1990). It contains many charged amino acid residues and is responsible for interactions with general transcription factors. One of the five evolutionary conserved regions is located in the transactivation domain (Soussi and May, 1996). This region (residues 12–23 of human p53) is implicated in interaction with mdm2 protein (Marston *et al.*, 1994; Picksley *et al.*, 1994). The latter negatively regulates p53 activity and is probably the main cellular switch which "turns off" p53 when p53 signalling pathway is not necessary any more.

The proline-rich domain was most recently defined as an autonomous functional region which is necessary for efficient growth suppression and receiving the antiproliferative signal initiated by Gas1 protein (Walker and Levine, 1996; Ruaro *et al.*, 1997). It contains five repeats of the amino acid sequence PXXP (P designating proline and X designating any amino acid). This motif is characteristic for interactions with proteins containing the SH3 domain and therefore can be a docking site of different factors which in turn can induce or suppress p53 activities. This region of the protein seems to be also involved in induction of apoptosis by p53 (Sakamuro *et al.*, 1997; Venot *et al.*, 1998) and is necessary for inhibition of papillomavirus replication (Lepik *et al.*, 1998). Recent studies indicate that a part of this region (residues 80–93) is implicated in regulation of the sequence-specific DNA binding of p53 (Hansen *et al.*, 1998; Müller-Tiemann *et al.*, 1998).

The central part of p53 (amino acid residues 100–300, named also "the core domain") is the sequence specific DNA binding domain (Bargonetti *et al.*, 1993; Halazonetis and Kandil, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). This region contains four of five evolutionary conserved domains of p53 and is the most frequently mutated region of p53 found in tumor cells. In addition to direct contact with DNA this domain plays crucial role in formation of overall conformation of the protein. Three-dimensional co-crystal structure of the DNA binding domain bound to its cognate site has been determined (Fig. 1; Cho *et al.*, 1994). The domain consists of a β sandwich, comprising two antiparallel β sheets, that serves as a scaffold for the structural elements at the DNA-protein interface. There are two components of these structural elements: a loop-sheet-helix motif that binds in the major groove and is involved in contacts with the

bases, and two large loops (L2 and L3) that interact so that a critical residue, Arg248 (in L3), makes contact with the minor groove of DNA in the A/T-rich region of the binding site. L2 and L3 interactions are stabilized by the zinc atom held in place by four metal-binding ligands, Cys176 and His179, which are in the L2 loop, and Cys238 and Cys242, within the L3 loop. Importantly, four evolutionary conserved regions comprise the L2 and L3 loops and the loop-sheet-helix motif that have critical roles in providing the structure, surfaces and residues that actually contact DNA. Based on crystal structure data, there are two major classes of naturally occurring mutations in p53: mutations in residues directly contacting DNA and those that disrupt the structural integrity of the domain. In both cases, p53 is not able bind to DNA, demonstrating that sequence-specific DNA binding is central to the normal functioning of p53 as a tumor suppressor.

The oligomerization domain is necessary to form p53 homo di- and tetramers. Although monomeric p53 retains some of wt protein activities (partial growth suppressor activity, activation of transcription form certain promoters, sequence-specific DNA binding to some binding sites), the tetrameric conformation of p53 is necessary to achieve fully functional protein (Shaulian *et al.*, 1993; Crook *et al.*, 1994; Sang *et al.*, 1994; Pellegata *et al.*, 1995). In cells heterozygous for p53 the heterooligomerization of mutated p53 with wt protein is the main way to inactivate the wt protein. Oligomerization domain of human p53 has been mapped between residues 326–355 (Wang *et al.*, 1994), three-dimensional structure of this fragment has been determined using nuclear magnetic resonance (Fig. 1; Lee *et al.*, 1994) and X-ray crystallography (Jeffrey *et al.*, 1995). Each p53 monomer is comprised of a β-strand (residues 326–333), a tight turn (Gly334) and a α-helix (residues 335–354). Consistent with its role as a critical hinge residue between the β strand and the α helix, Gly334 is conserved across the species (Soussi and May, 1996) and is one of the few oligomerization domain residues that has been found to be mutated in tumors (Greenblatt *et al.*, 1994). The tetramer can be described as a dimer of dimers, each primary dimer of which is formed by an antiparallel β-sheet and two antiparallel α-helices. Two of these dimers associate across second and distinct parallel helix-helix interface to form tetramer. The β strands are on the outside of tetramer and are not involved in dimer-dimer interactions.

The last 30 C-terminal amino acids of p53 are defined as a region which regulates sequence-specific DNA binding activity of the core domain. Under normal circumstances the p53 is in the "latent" form: its sequence-specific DNA binding activity is down-regulated. Modification of C-terminal domain removes the inhibitory effect from the core domain and sequence-specific DNA binding of the protein is activated. Activating modifications can be phosphorylation by casein kinase II or protein kinase C (Hupp *et al.*, 1992; Takenaka *et al.*, 1995), acetylation (Gu and Roeder, 1997) or glycosylation of C-terminus (Shaw *et al.*, 1996). DNA binding of p53 can be activated also by binding of

antibody pAb421 to the C-terminus or with deletion of the last 30 amino acids of the protein (Hupp *et al.*, 1992). Importantly, the carboxy-terminal domain of p53 recognizes certain types of damaged DNA including short single-stranded DNA molecules which also activate the sequence-specific DNA binding function of p53 (Bakalkin *et al.*, 1995; Jayaraman and Prives, 1995; Lee *et al.*, 1995; Reed *et al.*, 1995). Therefore the C-terminus can be a sensor which receives signal of DNA damage and responses to it by activating sequence-specific DNA binding of p53. One possible switch from the latent to the active state of p53 is induction of alternative splicing of p53 transcript. Alternatively spliced mRNA has been described in mice cells and it encodes p53 protein which is 9 amino acids shorter and has 17 different amino acids at the carboxyl terminus compared to "regularly spliced" form (Arai *et al.*, 1986; Kulesz-Martin *et al.*, 1994). This protein is permanently active for DNA binding (Wu *et al.*, 1994; Wolkowicz *et al.*, 1995).

2.1.2.2. Conformational flexibility

Although structures of the DNA binding and oligomerization domains of p53 are determined, the conformation of the whole molecule is not known. Little is known about the conformation of transactivation and proline-rich domains in the N-terminus and also about the linker region between DNA binding and oligomerization domains. The structure and intramolecular interactions of the C-terminal domain have been not determined either, but there is strong evidence that at least some parts of the molecule are capable of change their conformations. First, the C-terminal domain inhibits the sequence-specific DNA binding activity of the core domain and this inhibition is "removable" by many factors. Second, p53 has epitopes for some antibodies that can be accessible in certain cases but not in the other cases.

The conformational stages of p53 protein are mostly described by interactions with different antibodies. p53 reactive to pAb240 antibody is defined as a "mutant" conformation, because most of the mutated p53s have this epitope exposed. The "wild-type" conformation does not bind to pAb240, but reacts with pAb1620 and pAb246 antibodies. It has been proposed that wt p53 protein can switch between both conformations and this flexibility could be prerequisite for interacting with DNA. On the other hand, mutant p53s are locked into "mutant" conformation and have therefore lost its DNA binding activity (Milner, 1995). According to this model, it has been shown that wt p53 adopts "mutant-like" conformation when bound to DNA (Halazonetis *et al.*, 1993; Halazonetis and Kandil, 1993). As the DNA binding domain of p53 contains a zinc atom which interacts with L2 and L3 loops and stabilizes conformation of the core domain (Cho *et al.*, 1994), the "mutant" conformation of wt protein can be also induced by chelation of zinc or increasing concentration of copper (Hainaut *et al.*, 1995; Verhaegh *et al.*, 1997; Verhaegh *et al.*, 1998). The latter

probably competes with zinc in binding to p53 and disrupts the exact structure of the core domain.

2.1.3. Activities of p53

Very intensive studies of p53 during past years have revealed that many processes in the cell are directly or indirectly influenced by p53. This can reflect a central role of p53 in the regulation of cell life, but on the other hand, it is hard to imagine that one molecule can posses so many activities at the same time. At least some of the following activities have been tested *in vitro* only, therefore their relevance *in vivo* remains unclear. However, despite its many functions and important role in cancerogenesis, p53 is not essential protein in development, because at least some $p53^{-/-}$ mice develop and live normally — until they die due to tumors (Donehower *et al.*, 1992). This indicates that p53 is first of all needed for blocking the unregulated proliferation of cells.

The most pronounced property of p53 is acting as transcriptional regulator. It activates transcription of many genes, several of which are involved in mediation of p53-dependent cell cycle block in G_1 or G_2 phase. G_1 block is mostly achieved by activation of transcription of Waf1, a inhibitor of G_1 cyclin-dependent kinases (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a). G_2 block can be mediated, at least in some cell types, by activation of transcription of 14–3–3σ gene (Hermeking *et al.*, 1997). p53 also represses transcription from many cellular and viral promoters. The biological meaning of repression is unclear, but the ability of p53 to repress transcription correlates with its ability to induce apoptosis.

p53 induces apoptosis in some cell types. It is not clear whether transcriptional activation property of p53 is needed for this activity, but most probably both transcription dependent and independent mechanisms are involved in the induction of apoptosis. At least in some cells, transactivation competent p53 is more powerful inducer of apoptosis than its transactivation incompetent mutant. Genes like Bax and IGF-BP3, products of which influence the progression of apoptosis, are potential transcriptional targets of p53 (Buckbinder *et al.*, 1995; Miyashita and Reed, 1995). On the other hand, it is quite difficult to evaluate the role of p53 in induction of these genes as compared to other factors accompanying the ongoing of apoptosis. Clearly, p53 is not the only factor which regulates their transcription.

p53 inhibits both cellular and viral replication in *in vitro* and *in vivo* assays. In some circumstances the direct and indirect (through induction of cell cycle arrest or apoptosis) mechanisms of inhibition of replication are indistinguishable, but some recent works indicate that neither the cell cycle arrest nor apoptosis is responsible for this activity. Neither is the activation of transcrip-

tion by p53 involved, because p53 inhibits cellular DNA replication in transcription free *Xenopus* egg extracts *in vitro* and transactivation incompetent mutant of p53 inhibits papillomavirus replication *in vivo* (Cox *et al.*, 1995; Lepik *et al.*, 1998).

The loss of wt p53 activity contributes to genomic DNA amplification, indicating possible role of p53 in the control of genome integrity (Livingstone *et al.*, 1992; Yin *et al.*, 1992). p53 interacts with DNA repair (and transcription) factor TFIIH, down-regulating its helicase activity, and with Rad51 protein, which is involved in DNA recombination (Wang *et al.*, 1995a; Leveillard *et al.*, 1996; Stürzbecher *et al.*, 1996). Also, p53 can catalyze reannealing of single-stranded complementary nucleic acids *in vitro* (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994). Based on these facts it has been proposed that p53 could be directly involved in DNA repair and recombination processes.

It has been reported that p53 can repress translation of at least two mRNAs: its own and CDK4 mRNA (Ewen *et al.*, 1995; Mosner *et al.*, 1995). Repression of its own mRNA can be involved in keeping the p53 protein in the low level and its induction after DNA damage, repression of CDK4 translation may be involved in regulation of cell cycle by p53.

There have been two additional enzymatic activities of p53 described: 1) p53 possesses intrinsic 3'→5' exonuclease activity *in vitro* (Mummenbrauer *et al.*, 1996), which can be involved in regulation of DNA repair or replication by p53. 2) p53 is a autoprotease that can cleave itself from both N- and C-terminus and this can be involved in regulation of DNA binding, transactivation or other activities of p53 (Molinari *et al.*, 1996; Okorokov *et al.*, 1997).

2.2. p53 as a modulator of transcription

2.2.1. Interactions with general transcription machinery

Transcriptional activation by p53 is based on its ability to bind sequence-specifically to DNA and recruit the general transcription factors to the promoters located near to its binding site. TATA-box binding protein (TBP) was the first identified protein among the general transcription factors which interact with p53 (Seto *et al.*, 1992; Liu *et al.*, 1993; Martin *et al.*, 1993; Truant *et al.*, 1993). The TBP binding domain on p53 was mapped to transactivating domain (Liu *et al.*, 1993), later studies revealed that the second TBP binding site is located in the C-terminus of p53 (Horikoshi *et al.*, 1995). TBP is capable of interacting with p53 also when bound to its cognate DNA site (Martin *et al.*, 1993) and p53 also cooperates with either TBP or TFIID when binding to DNA fragment containing both TATA-box and p53 binding site (Chen *et al.*, 1993). Although direct interaction between TBP and p53 has been well documented, there is a doubt about the physiological relevance of this interaction: 1) p53 double

mutant at amino acids Leu22 and Trp23 is not able to activate transcription, although it retains nearly full TBP binding activity (Lin *et al.*, 1994). 2) Mutations in TBP, which disrupt its association with p53 *in vitro*, do not disrupt its transcriptional response to p53 activation domain *in vivo* (Tansey and Herr, 1995). It is more likely that the true target of p53 is the entire TFIID complex, consisting of TBP and associated factors, in which the interaction with TBP is possible but not critical for transactivation. Indeed, p53 interacts also with TBP-associated factors, $TAF_{II}40$ and $TAF_{II}60$, from *Drosophila* cells and $TAF_{II}31$ from human cells and these interactions are disrupted by mutations at amino acids 22 and 23 in p53 protein (Lu and Levine, 1995; Thut *et al.*, 1995).

p53 interacts also with components of TFIIH complex: ERCC2 (XPD), ERCC3 (XPB) and p62 (Xiao *et al.*, 1994; Wang *et al.*, 1995a; Leveillard *et al.*, 1996). Both N and C termini of p53 are involved in these interactions. TFIIH is multifunctional complex which is involved in initiation of transcription, in cell cycle regulation and in DNA repair (Seroz *et al.*, 1995). The complex has two enzymatic activities: ERCC2 and ERCC3 are helicases that are essential components of the cellular DNA repair machinery (Schaeffer *et al.*, 1993; Schaeffer *et al.*, 1994), and the kinase complex CAK (CDK-activating kinase) phosphorylates C-terminal domain of RNA Pol II large subunit and may thereby regulate initiation and elongation of transcription (Lu *et al.*, 1992). Functional meaning of p53-TFIIH interaction is unclear in respect to both transcriptional regulation and regulation of TFIIH-mediated nucleotide excision repair (NER). It has been shown that p53 does not affect NER *in vitro* (Leveillard *et al.*, 1996), although it inhibits activity of both helicases of the TFIIH complex (Wang *et al.*, 1995a; Leveillard *et al.*, 1996). Two recent studies propose that TFIIH-p53 interaction can be important first of all for regulation of p53 itself. TFIIH associated kinase complex CAK (consisting from three subunits: CDK7, cyclin H and $p36^{MAT1}$) also interacts with p53 and phosphorylates its N- or C-terminus (Ko *et al.*, 1997; Lu *et al.*, 1997). The functional relevance of these phosphorylations remains to be determined, but it has been proposed that phosphorylation of C-terminus by CAK activates sequence-specific DNA binding of p53 (Lu *et al.*, 1997). However, TFIIH as a component of the DNA repair complex can be a upstream messenger that "informs" p53 about DNA damage.

In addition to interactions with the general transcription factors, p53 binds also cellular proteins p300 and CBP (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997; Scolnick *et al.*, 1997). Both proteins possess histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). The acetylation of histones is thought to be involved in destabilization and restructuring of nucleosomes, which is likely a crucial event for accessibility of transcription factors to DNA templates. Therefore it is possible that the first step of activation of transcription by p53 is to bring histone acetyltransferases near to the particular promoter. After being relieved from histones, promoter is

ready to bind basal transcription factors, TFIID for example. In accordance with this model, co-expression of CBP or p300 with p53 enhances p53-dependent activation of transcription (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997). The binding site of CBP is mapped to transactivation domain of wt p53, mutations at residues Leu22 and Trp23 disrupt the interaction (Gu *et al.*, 1997; Scolnick *et al.*, 1997). The interaction between p300 and p53 has also importance for p53 activity: p300 acetylates p53 at the C-terminal regulatory domain, preferentially at Lys373 and Lys382, which in turn leads to the activation of DNA binding of p53 (Gu and Roeder, 1997).

2.2.2. Interactions with DNA

p53 is able to bind DNA in two different ways: sequence-specifically and non-specifically. Sequence-specific binding is required first of all for transcriptional activation by p53, non-specific interaction is involved in activation of sequence-specific DNA binding, participates in recognition of damaged DNA and is needed for other activities (reannealing of nucleic acids, exonuclease activity). The core domain of p53 (amino acids 100–300) is responsible for sequence-specific DNA binding, whereas non-specific binding is intrinsic property of both core and C-terminal regulatory domains.

p53 binds specifically 20 base pair DNA sequence containing two copies of motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by 0–13 bases (El-Deiry *et al.*, 1992; Funk *et al.*, 1992). Regardless to the fact that this sequence allows very broad variation, the natural p53 binding sites found in promoters of target genes do not meet the whole criteria for this consensus. There is at least one mismatch in every real binding site (Table 1). On the other hand, sometimes there are two p53 binding sites in the vicinity of target gene promoter, which can compensate deficiency of each individual site. Moreover, not all sequences, which perfectly match with consensus, are bound by p53 *in vitro* (Halazonetis *et al.*, 1993). All these circumstances (including variation of space between two decamers in the consensus) make it very difficult to predict the real functional p53 binding sites. It is estimated that the human genome could contain approximately 200–300 of such sites (Tokino *et al.*, 1994).

The consensus site can be viewed as a structure of four quarter-site repeats: 5'-PuPuPuC(A/T)-3' considering that the rest of a half site [5'-(A/T)GPyPyPy-3'] forms the same sequence on the complementary strand of DNA. Each pentameric repeat binds to single monomer of tetrameric p53 protein. Monomeric p53 molecules, which have lost their oligomerization capacity, bind to the consensus site as four monomers only, indicating that this binding is strongly cooperative (Balagurumoorthy *et al.*, 1995; Wang *et al.*, 1995b). Binding of p53 to DNA causes considerable bending of DNA chain. The bending angle

varies between different sites, but is greater (up to 50°) in high affinity sites. In particular, response elements having a CATG sequence at the junction of two consensus pentamers in each half-site favor highly bent complexes, whereas response elements having CTTG bases at this position are less bent (Nagaich *et al.*, 1997).

Sequence-nonspecific DNA binding through the C-terminal domain of p53 has several implications. When bound to short single-stranded DNA, the C-terminal domain "rescues" the sequence-specific DNA binding activity of the core domain (Jayaraman and Prives, 1995). One explanation of this activation is that p53 is tightly bound to cellular DNA through its C-terminal domain and short ssDNA releases p53 by competing for binding to C-terminal domain. When bound to ssDNA, p53 is "relatively free" to move and make interactions through the core domain. According to this model, it has been shown that large DNA molecules actually inhibit intrinsic sequence-specific DNA binding activity of purified p53, whereas short DNA molecules can overcome this

Table 1. Genomic p53 binding sites. Capital letters indicate bases matching with the consensus, bold letters indicate mismatches, letters in *italics* indicate spacer sequence between consensus decamers.

Name	Sequence			Reference
Consensus	PuPuPuC(A/T)(A/T)GPyPyPy$N_{0\text{-}13}$PuPuPuC(A/T)(A/T)GPyPyPy			(El-Deiry *et al.*, 1992)
Waf1 (human)	GAACATGTCC		**c**AACATGTT**g**	(El-Deiry *et al.*, 1993)
Mdm2 (1) (mouse) (2)	GG**t**CAAGTT**g** AG**ct**AAGTCC		GGACA**c**GTCC **t**GACATGTCT	(Juven *et al.*, 1993; Wu *et al.*, 1993)
Mdm2 (1) (human) (2)	GG**t**CAAGTTC GA**tt**AAGTCC		AGACA**c**GTCC **t**GACTTGTCT	(Zauberman *et al.*, 1995a)
GADD45 (human)	GAACATGTCT		AAGCATGCT**g**	(Kastan *et al.*, 1992)
Cyclin G (mouse)	AGAC**c**TGCCC		GGGCAAGCCT	(Okamoto and Beach, 1994)
Cyclin G (1) (rat) (2)	AGAC**c**TGCCC A**c**GCAAGCCC		GGGCAAGCCT GGGCTAGTCT	(Zauberman *et al.*, 1995b)
MKC (mouse)	t**g**GCAAGCCT	*A*	**t**GACATG**g**CC	(Zambetti *et al.*, 1992)
Bax (human)	**tc**ACAAGTT**a**	*G*	AGACAAGCCT	(Miyashita and Reed, 1995)
PCNA (human)	A**c**A**t**ATGCCC		GGACTTGTTC	(Shivakumar *et al.*, 1995)
RGC (human)	GGACTTGCCT		GG**c**CTTGCCT	(Kern *et al.*, 1991)
IGF-BP3 (human) boxA boxB	AAACAAGCC**a** GGGCAAG**a**CC	*C*	**c**AACATGCTT **tGc**CAAGCCT	(Buckbinder *et al.*, 1995)

inhibition (Anderson *et al.*, 1997). This type of p53 activation can occur in cells during excision repair after UV-dependent DNA damage: the human excinuclease complex first recognizes UV damaged DNA and then introduces single strand incisions at positions 3' and 5' to the mismatch. A product of the subsequent repair of the lesion is the release of single-stranded 29-mer DNA (Huang *et al.*, 1992), which is a good candidate for activating p53. Another function of DNA binding by C-terminal domain is that it specifically recognizes and binds to some types of damaged DNA. p53 (and its C-terminal domain) binds to insertion/deletion mismatches in double-stranded DNA (Lee *et al.*, 1995), to the ends of single-stranded DNA (Bakalkin *et al.*, 1994; Bakalkin *et al.*, 1995; Selivanova *et al.*, 1996), and to DNA damages introduced enzymatically (Dnase I, produces mostly single-stranded nicks) or by ionizing radiation (causes variety of strand breaks; Reed *et al.*, 1995). These interactions may be involved in activation of sequence-specific DNA binding or stability of p53. p53 also possesses activity of reannealing complementary nucleic acids. Non-specific DNA binding by the C-terminal domain of the protein is needed for this activity (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994; Bakalkin *et al.*, 1995; Wu *et al.*, 1995).

2.2.3. Target genes

Several target genes directly activated by p53 have been identified. These include muscle creatine kinase (Zambetti *et al.*, 1992), Mdm2 (Barak *et al.*, 1993), WAF1 (El-Deiry *et al.*, 1993), GADD45 (Kastan *et al.*, 1992), cyclin G (Okamoto and Beach, 1994), Bax (Miyashita and Reed, 1995), IGF-BP3 (Buckbinder *et al.*, 1995), "PIG group" genes (Polyak *et al.*, 1997), 14–3–3σ (Hermeking *et al.*, 1997) and others. Although p53 can activate transcription of these genes, they also have p53-independent ways for regulation of their expression. There are clear biological outputs associated with induction of some p53 responsive genes, whereas the relevance of others remains obscure.

2.2.3.1. Genes associated with cell cycle regulation.

Waf1 (also named p21 or Cip1) is the best studied p53 response gene. The protein encoded by this gene forms part of a quaternary complex with cyclin/CDKs and the DNA polymerase processivity factor PCNA found in normal cells (Xiong *et al.*, 1993b). At high protein concentrations, Waf1 inhibits the function of CDKs, particularly of those which function during the G_1 phase of the cell cycle (Harper *et al.*, 1993; Xiong *et al.*, 1993a). In response to irradiation, p53-dependent G_1 arrest is mediated, at least in part, through the induction of Waf1 by p53 (El-Deiry *et al.*, 1994). At this point p53 pathway meets

another tumor suppressor gene: Rb. Inhibition of CDKs leads to the accumulation of hypophosphorylated Rb which binds (and therefore inactivates) E2F family transcription factors. E2F in turn activates number of genes required to initiate or propagate the S phase of the cell cycle. Waf1 has also been implicated in regulating DNA replication. Waf1 directly interacts with PCNA and inhibits PCNA-dependent DNA replication (but not DNA repair synthesis) in *in vitro* systems (Flores-Rozas *et al.*, 1994; Li *et al.*, 1994; Waga *et al.*, 1994). Therefore, Waf1 may block chain elongation during DNA synthesis through its interaction with PCNA while permitting PCNA-dependent nucleotide excision repair to occur. However, cells derived from Waf1 null mice retain some ability to arrest in G_1 in response to DNA damage (Brugarolas *et al.*, 1995; Deng *et al.*, 1995), indicating that Waf1 is not entirely responsive for antiproliferative effect of p53. The DNA sequence in Waf1 gene responsible for binding p53 is located 2.4 kb upstream of TATA box (El-Deiry *et al.*, 1993).

GADD (growth arrest and DNA damage) genes were initially isolated on the basis of their induction after DNA damage in mammalian cells (Fornace *et al.*, 1988; Fornace *et al.*, 1989). GADD45 gene is induced after DNA damage in many types of cells in p53 dependent manner (Kastan *et al.*, 1992). The GADD45 protein was reported to interact with the PCNA and to inhibit the entry of cells into S phase of cell cycle upon re-stimulation with serum (Smith *et al.*, 1994). Overexpression of GADD45 protein also results in the inhibition of colony formation (Zhan *et al.*, 1994). p53 binding site is located in the third intron of GADD45 gene (Kastan *et al.*, 1992). p53 contributes to activation of GADD45 gene even in absence of direct binding to DNA. In this case, the WT1 binding site in front of GADD45 gene is required and p53 activation effect is achieved by interaction with WT1 protein (Zhan *et al.*, 1998).

Cyclin G gene is strongly induced in p53-dependent manner after DNA damage (Okamoto and Beach, 1994; Zauberman *et al.*, 1995b). The function of the protein is unknown, therefore its participation in the cell cycle regulation is hypothetical. Cyclin G has been shown to interact with B' subunit of protein phosphatase 2A (Okamoto *et al.*, 1996). p53 binding site was found 1.5 kb upstream of the coding sequence of mouse cyclin G gene (Okamoto and Beach, 1994). Rat cyclin G gene contains two p53 binding sites: 250 nucleotides upstream of transcriptional start site and in the first intron (Zauberman *et al.*, 1995b).

14–3–3σ was recently identified as a p53 response gene (Hermeking *et al.*, 1997). Induction of 14–3–3σ can be one mechanism how p53 mediates cell cycle block in G_2 phase. Overexpression of the 14–3–3σ causes the block of cell proliferation and accumulation of cells in G_2/M phase. The possible mechanism of 14–3–3σ in cell cycle regulation is that it binds to Cdc25C phosphatase, blocks its entry into cell nucleus and therefore abolishes dephosphorylation of Cdc2, a cyclin-dependent kinase required for entry into mitosis

(Peng *et al.*, 1997). A functional p53 biding site is located 1.8 kb upstream of transcription initiation site of 14–3–3σ gene (Hermeking *et al.*, 1997).

2.2.3.2. Genes associated with apoptosis

The Bax gene encodes a protein with homology to the survival factor Bcl-2. It is able to homodimerize and to form heterodimers with Bcl-2 protein. Site-directed mutagenesis of Bcl-2 protein indicated that Bcl-2 must bind to Bax protein to exert its apoptosis-suppressing activity (Yin *et al.*, 1994). On the other hand Bax homodimers are potent inducers of apoptosis. Therefore relative levels of these two proteins may dictate whether a cell is susceptible to apoptosis (Oltvai *et al.*, 1993). p53 activates transcription of Bax and therefore induces unbalance between levels of Bcl-2 and Bax proteins which in turn can be one component needed for the induction of apoptosis. Binding site for p53 is located 70 nucleotides upstream of TATA box of human Bax gene (Miyashita and Reed, 1995).

IGF-BP3 (insulin-like growth factor-binding protein-3) blocks the IGF mitotic signalling pathway by binding to IGF and preventing its interaction with its receptor. Thus, blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells. There are three p53 binding sites in the IGF-BP3 gene: in intron 1 (Box A) and in intron 2 (Box B; Buckbinder *et al.*, 1995). The third functional binding element was found by computer analysis and is located 70 nucleotides upstream of TATA box. Functional significance of this site was also confirmed (Bourdon *et al.*, 1997).

"PIG (p53-induced genes) group" genes were found to be induced during onset of p53-dependent apoptosis. 14 transcripts were found to be markedly increased, many of these genes were predicted to encode proteins that could generate or respond to oxidative stress (Polyak *et al.*, 1997). From this group of genes the PIG3 transcript was studied more detail. PIG3 is a novel gene highly related to TED2, a plant NADPH oxidoreductase. Recent data show that the proline-rich region of p53 is necessary for activation of PIG3 gene (Venot *et al.*, 1998). p53 responsive element able to bind p53 is located 308 nucleotides upstream of transcription start site.

p53-dependent activation of apoptosis-associated Fas/APO-1 and Killer/DR5 genes has also been reported (Owen-Schaub *et al.*, 1995; Wu *et al.*, 1997), but p53 response elements of these genes have not been identified. Therefore, there remains the possibility that these two genes are not direct targets of p53.

2.2.3.3. Mdm2: keeping p53 under control

The mdm2 gene encodes a protein which complexes with p53 and inhibits its transcriptional activation ability (Momand *et al.*, 1992; Oliner *et al.*, 1993). The mdm2 gene itself is a transcriptional target of p53 and is activated in response to UV irradiation, thus implying an autoregulatory feedback loop between p53 and mdm2 (Barak *et al.*, 1993; Perry *et al.*, 1993; Wu *et al.*, 1993). Two promoters of mdm2 gene were characterized: the first one is located upstream of the gene and is expressed constitutively, whereas the second one is present inside the first intron and is controlled by p53 through two adjacent p53 binding sites (Juven *et al.*, 1993; Wu *et al.*, 1993; Barak *et al.*, 1994; Zauberman *et al.*, 1995a).

Fine mapping with short synthetic peptides revealed that the mdm2 binding site on p53 spans amino acids 18–23 (Picksley *et al.*, 1994), which coincide with the first evolutionary conserved domain of p53 (Soussi and May, 1996). Residues Leu14, Phe19, Leu22 and Trp23 of p53 were identified by mutational approach as critical residues making contact with mdm2 (Lin *et al.*, 1994). Residues Leu22 and Trp23 are also required for transcriptional activation and binding to $TAF_{II}31$, demonstrating an overlap between the transactivation domain and the mdm2 binding domain of p53 (Lin *et al.*, 1994; Lu and Levine, 1995). In 1996 the three-dimensional structure of a 109 amino acid residues long amino-terminal domain of *Xenopus laevis* mdm2 bound to the 15 amino acid residues long transactivation domain of p53 was determined (Kussie *et al.*, 1996). This revealed that mdm2 has a deep hydrophobic cleft on which the p53 peptide binds as an amphipathic α-helix. Residues Phe19, Trp23 and Leu26 of p53 stabilize these hydrophobic interactions between p53 and mdm2 in this cleft.

The functional significance of mdm2-dependent inactivation of p53 was demonstrated when it was attempted to produce mdm2 knockout mice. The first result was that mdm2-null genotype leads to embryonic lethality. $Mdm2^{+/-}$ heterozygote mice are viable, develop normally and are fertile but interbreeding of $mdm2^{+/-}$ heterozygotes failed to result in offspring with the $mdm2^{-/-}$ genotype. Crossing $mdm2^{+/-}$ mice with $p53^{+/-}$ or $p53^{-/-}$ mice resulted progeny that were homozygous for both mdm2 and p53 null alleles. No mice were recovered that were null for mdm2 at wt or heterozygous p53 background (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). These results demonstrate that primary developmental role of mdm2 is to negatively regulate wt p53 function. More detailed investigation of mdm2 inhibitory role on p53 revealed that when p53 is bound by mdm2, it is targeted for destruction by the ubiquitin-dependent proteosome pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Binding of mdm2 to p53 is necessary but not sufficient for degradation of p53: deletion mutant of mdm2 interacting with p53 and inhibiting p53-induced cell cycle arrest but lacking the C-terminal part of protein, is not able to induce degradation of p53 (Kubbutat *et al.*, 1997). At the conditions of genotoxic stress the p53 activity is

continuously needed, but this causes accumulation of mdm2, which in turn leads to the degradation of p53. Therefore it is reasonable to expect that mdm2-p53 interaction is somehow regulated. There are several potential phosphorylation sites nearby mdm2 binding site on p53. It has been shown that phosphorylation of p53 residue Ser15 weakens p53-mdm2 interaction and DNA damage induces p53 phosphorylation at this site (Shieh *et al.*, 1997; Siliciano *et al.*, 1997). Therefore phosphorylation at Ser15 can be a mechanism causing the protection of p53 from mdm2-dependent degradation. However, recent *in vitro* data show that phosphorylation of both Ser15 and Ser37 are needed for disruption of mdm2-p53 interaction. p53-TFIID interaction is disrupted by single phosphorylation at Ser15, but double phosphorylation at Ser15 and Ser37 restores TFIID binding activity of p53 (Pise-Masison *et al.*, 1998). Very attractive candidate for phosphorylation of these sites is the DNA-dependent protein kinase (DNA-PK), which requires DNA double-strand breaks or other discontinuities in DNA for activity (Gottlieb and Jackson, 1993; Morozov *et al.*, 1994). Indeed, p53 is substrate of DNA-PK *in vitro* (Lees-Miller *et al.*, 1990; Lees-Miller *et al.*, 1992). Mdm2 is also substrate of DNA-PK and its phosphorylation by DNA-PK also prevents mdm2-p53 interaction (Mayo *et al.*, 1997). On the other hand, DNA-PK activity is not required for accumulation of p53, indicating that alternative pathway for regulation of p53-mdm2 interaction may exist (Rathmell *et al.*, 1997).

2.2.4. Repression of transcription

In addition to the activation of transcription from some promoters, p53 is also potent repressor of others. Whereas the transcriptional activation by p53 needs the functional p53 binding site vicinity of the promoter, the repression seems not generally depend on any DNA sequences. This also makes it more difficult to determine whether the particular promoter is a direct target of p53 or is its repression simply a secondary effect of p53. As p53 mediates many processes in the cell, including cell cycle arrest and apoptosis, it is likely that some promoters reported to be repressed by p53 are in fact sensitive for cell cycle progression or onset of apoptosis or are dependent on proteins which are influenced by p53. In addition: thus far the ability of p53 to repress transcription correlates to its ability to induce apoptosis. Nevertheless, several promoters are down-regulated by wt p53. These include many viral promoters like SV40 IE promoter and enhancer, RSV-LTR, human CMV IE promoter, HIV-LTR, HTLV-TypeI-LTR, HSV thymidine kinase promoter, HSV UL9 gene promoter and HPV 18 and HPV 16 LCR (Subler *et al.*, 1992; Jackson *et al.*, 1993; Desaintes *et al.*, 1995). Also hepatitis B virus (HPV) enhancer is repressed by p53 and in this case p53 binds sequence-specifically to the enhancer region.

However, the proximal sequence of HPV enhancer, containing binding site for RFX1 and c-Abl complex, is required to convert p53 to transcriptional repressor (Ori *et al.*, 1998). Cellular genes repressed by p53 include Bcl-2 (Miyashita *et al.*, 1994), rat brain creatine kinase (Zhao *et al.*, 1994), microtubule associated protein 4 (Murphy *et al.*, 1996), hsp70 (Agoff *et al.*, 1993), Ets-1 and Ets-2 (Iotsova *et al.*, 1996), c-Myc (Ragimov *et al.*, 1993), DP1 (Gopalkrishnan *et al.*, 1998), H19 (Dugimont *et al.*, 1998) and others. Earlier investigations suggested that p53 specifically represses TATA-mediated but not initiator-mediated transcription (Mack *et al.*, 1993). More recent studies, however, show that also many TATA-less promoters, like Ets-1, Ets-2, H19 and DP1, are efficiently repressed by p53 (Iotsova *et al.*, 1996; Dugimont *et al.*, 1998; Gopalkrishnan *et al.*, 1998). It is also reported that some promoters directed by RNA polymerase III are repressed by p53 (Chesnokov *et al.*, 1996; Cairns and White, 1998).

Although monomeric p53 is able to activate transcription, it is not able to repress promoters (Crook *et al.*, 1994; Sang *et al.*, 1994; Subler *et al.*, 1994; Shaulian *et al.*, 1995). It has been demonstrated that phosphorylation of p53 from the casein kinase II site (Ser386 on mouse p53) is required for efficient repression of transcription (Hall *et al.*, 1996). On the other hand, phosphorylation of the same site essentially increases stability of p53 tetramerization *in vitro* (Sakaguchi *et al.*, 1997). Thus, it can be the same thing: p53 must be tetrameric to repress transcription. One proposed mechanism how p53 represses transcription is through squelching of transcription factors. This is based on fact that p53 unable to interact with components of TFIID complex is also unable to repress transcription (Chesnokov *et al.*, 1996; Roemer and Mueller-Lantzsch, 1996).

2.3. Regulation of p53 activity

2.3.1.Post-translational modifications of p53

The N- and C-termini of p53 are post-translationally modified in multiple sites by phosphorylation, O-glycosylation and acetylation. The last two modifications were discovered relatively recently and in both cases the sequence-specific DNA binding of p53 is activated. Glycosylation of p53 prevents its binding to antibody pAb421 (Shaw *et al.*, 1996) indicating that the site of this modification lies near to pAb421 epitope (residues 371–381). Acetylation of p53 occurs also in the same region: lysines 373 and 382 (but also lysines 370, 372 and 381 in lesser extent) are acetylated *in vitro*. In the case of acetylation, the ability of p53 to bind to pAb421 is not affected (Gu and Roeder, 1997). Whether and how the modifications of this region are regulated (or can they occur simultaneously) is not clear. The problem is even more complex, because

also serines 376 and 378 are phosphorylated by PKC (see below). It is possible that each of these modifications is induced in different occasions and by different factors, but give the same result in respect of p53 activity. Nevertheless, all modifications of this region (and binding to antibody) stimulate the sequence-specific DNA binding of p53, indicating its role in regulation of p53. It is proposed that this part of p53 interacts with the core domain inhibiting its DNA binding activity. Modification of C-terminus disrupts this association and makes thus possible the sequence-specific interaction of p53 with DNA.

p53 is phosphorylated by various kinases and several studies have revealed how it can regulate activities of p53. The N-terminal region of p53 appeared to be the most extensively phosphorylated part of the protein and target for various kinases. Phosphorylation by Casein I-like kinase *in vitro* has been shown at Ser4, 6 and 9 of mouse p53 (Milne *et al.*, 1992). Based on sequence homology, phosphorylation of human p53 on Ser6 and 9 by the same kinase can be predicted. DNA-PK phosphorylates serines 15 and 37 of human p53 (Lees-Miller *et al.*, 1992). Serine 15 of p53 becomes phosphorylated following DNA damage induced by either ionizing or ultraviolet radiation and this phosphorylation leads to reduced interaction of p53 with its negative regulator, mdm2 oncoprotein (Shieh *et al.*, 1997; Siliciano *et al.*, 1997). Therefore, phosphorylation of Ser15 (by DNA-PK or some other kinase with similar specificity) may induce transactivation activity of p53 and also lead to stabilization of the protein. JNK1, JNK2 and JNK3 kinases, activity of which is induced by UV radiation, can phosphorylate Ser34 of mouse p53 (Milne *et al.*, 1995; Hu *et al.*, 1997). According to sequence homology, Ser37 of human p53 can be phosphorylated by these enzymes. A related kinase, MAP-kinase, was found to phosphorylate mouse p53 on the Thr73 and Thr83 (Milne *et al.*, 1994), however, no homologs of these sites seem to be present in human p53. So far, no correlation is found between activity of p53 and its phosphorylation by MAP and JNK kinases.

The C-terminus of p53 is also phosphorylated by several kinases. Serine 315 of human p53 is phosphorylated by Cdc2 (Bischoff *et al.*, 1990; Stürzbecher *et al.*, 1990). More detailed investigation indicated that cyclin B/Cdc2 and cyclin A/Cdc2 efficiently phosphorylate this site *in vitro*, whereas p53 was only poorly phosphorylated by cyclin D/CDK4 and cyclin E/CDK2 complexes. In addition, phosphorylation of Ser315 strongly increases the binding of p53 to the cognate consensus sequence which is present in the Waf1 or Gadd45 gene, while the binding to RGC, MCK and SV40 consensus sequences was hardly influenced (Wang and Prives, 1995). Therefore, phosphorylation of Ser315 could induce selective, sequence-specific, DNA binding of p53.

Protein kinase C (PKC) phosphorylates p53 from multiple sites *in vitro*, but all sites are located in the vicinity of pAb421 epitope (aa 371–381). Serines 376 and 378 of human p53 and corresponding serines (370 and 372) of murine p53 have been clearly identified as sites phosphorylated by PKC (Baudier *et al.*,

1992; Takenaka *et al.*, 1995; Milne *et al.*, 1996; Delphin *et al.*, 1997). Phosphorylation by PKC masks pAb421 epitope on p53 and also induces sequence-specific DNA binding activity of p53 *in vitro* (Takenaka *et al.*, 1995). *In vivo* situation, however, can be different: in several cell types most of the p53 is phosphorylated at PKC site Ser376 (this protein is not recognized by pAb421 antibody). After induction of p53 activity by ionizing radiation, Ser376 is dephosphorylated, but Ser378 remains phosphorylated. This phosphorylation pattern forms consensus site for binding to 14–3–3 proteins, which in turn activate sequence-specific DNA binding of p53 *in vitro* (Waterman *et al.*, 1998). This allows to propose that 14–3–3 proteins can be pAb421-like natural activators of p53, association of which with p53 is regulated by phosphorylation/dephosphorylation at PKC sites.

The serine residue at position 392 of human p53 (equivalent to mouse Ser386) was found to be phosphorylated by casein kinase II (CKII; Meek *et al.*, 1990) and this phosphorylation leads to the activation of sequence-specific DNA binding of p53 (Hupp *et al.*, 1992). The biological meaning of phosphorylation of Ser392 is not fully understood. Some studies indicate that phosphorylation of this site is not needed for wt p53 activities (Fiscella *et al.*, 1994; Rolley and Milner, 1994), whereas others show that mutation of CKII site reduces p53-dependent transcriptional repression (Hall *et al.*, 1996) and activation (Hao *et al.*, 1996). Nevertheless, biochemical studies indicated that phosphorylation of Ser392 strongly stabilizes p53 tetramer formation *in vitro* (Sakaguchi *et al.*, 1997). It is possible that in normal circumstances (where p53 is expressed at very low concentration), the tetramerization of protein is subject of regulation indeed. Tetrameric p53 in turn, is more efficient for sequence-specific DNA binding than its monomeric form. This makes it very difficult to evaluate biological effect of mutations of phosphorylation site in assays, where p53 protein is overexpressed (which can compensate lower stability of tetramers).

In addition, p53 can be phosphorylated from N-terminus by Raf kinase *in vitro* (Jamal and Ziff, 1995), from N- or C-terminus by TFIIH-associated kinase complex CAK (Ko *et al.*, 1997; Lu *et al.*, 1997) and also by protein kinase A (Adler *et al.*, 1997). In these cases, exact target residues for phosphorylation are not known.

2.3.2. Cellular modulators of p53 activity

Activity of p53 is regulated also by many different protein-protein interactions. Whereas viral proteins usually cause inactivation of p53, cellular proteins are both positive and negative regulators of p53. The possible roles of mdm2, p300 and 14–3–3 in regulation of p53 were discussed earlier, but in addition to these,

there are several other proteins that have influence on p53 activities in the cell. Some, but not all, of them can physically interact with p53, while others show just co-operative effect in activation of p53-dependent transcription or DNA binding.

The Wilms' tumor-suppressor gene product WT1 has been shown to associate with p53 when both are overexpressed in the same cell (Maheswaran *et al.*, 1993). Co-expression of p53 and WT1 results in higher steady-state levels of p53, an increased level of p53 binding to sequence-specific DNA targets, and an enhanced transcriptional activity of p53, but reduces p53-mediated apoptosis (Maheswaran *et al.*, 1995). As WT1 is transcription factor itself, the enhancement of p53-dependent transcription can be achieved at higher concentration of transactivation domains, when WT1 is localized in the vicinity of promoter through interaction with DNA-bound p53. The same scheme of co-operation exists also in opposite way. In the case of GADD45 promoter, p53 can contribute to activation of transcription in two ways: binding directly to its cognate DNA sequence in the third intron of the gene (Kastan *et al.*, 1992) and by interacting with WT1 protein, binding site of which is in front of the promoter (Zhan *et al.*, 1998).

p53 interacts with two components of E2F transcription factors: DP1 and E2F1 and this interaction inhibits both the p53-dependent and E2F-dependent transcription (O'Connor *et al.*, 1995; Sorensen *et al.*, 1996). p53 and E2F1 proteins cooperate to mediate apoptosis (Wu and Levine, 1994; Kowalik *et al.*, 1998), which is probably the result of two conflicting signals: p53 induces cell cycle block in G_1 phase, but E2F1 can induce quiescent cells to enter S phase (Johnson *et al.*, 1993). Expression of E2F1 also induces accumulation of p53 (Kowalik *et al.*, 1998).

There are two other transcription factors which have been reported to interact with p53 and enhance its transactivation ability: BRCA1, which binds to C-terminus of p53 and enhances both p53-dependent transactivation and apoptosis (Ouchi *et al.*, 1998; Zhang *et al.*, 1998), and hypoxia-inducible factor 1α (HIF-1α), which also induces stabilization of p53 (An *et al.*, 1998). In addition, a putative tumor suppressor protein $p33^{ING1}$ interacts with p53, enhances p53-dependent transcription and is needed for efficient growth suppression by p53 (Garkavtsev *et al.*, 1998). Also c-Abl tyrosine kinase binds to p53 and increases its transactivation activity. In this case, the kinase activity of c-Abl is not needed, because a mutant of c-Abl deficient in kinase activity also stimulates p53. Activation of p53 depends on physical interaction between c-Abl and p53, which is mediated by proline-rich SH3 domain of c-Abl and probably involves proline-rich region of p53 (Goga *et al.*, 1995). The region of p53 near to proline-rich domain was recently reported to be involved in regulation of its DNA binding activity (Hansen *et al.*, 1998; Müller-Tiemann *et al.*, 1998). It is therefore possible that the stimulating effect of c-Abl can be achieved by activation of sequence-specific DNA binding of p53.

Ref-1 and HMG-1 proteins were found to activate sequence-specific DNA binding of p53, when fractions of nuclear extract of HeLa cells were tested for activation of p53-dependent transcription and DNA binding (Jayaraman *et al.*, 1997; Jayaraman *et al.*, 1998). Both proteins are quite nonspecific "helpers", which activate DNA binding of various other proteins. Ref-1 is a dual function protein which can both regulate the redox state of a number proteins and function as a DNA repair apurinic/apyrimidinic endonuclease (Demple *et al.*, 1991; Xanthoudakis and Curran, 1992; Xanthoudakis *et al.*, 1992). As DNA binding activity of p53 depends on its redox state (reduced p53 binds efficiently, while oxidized p53 binds very poorly; Hainaut and Milner, 1993), the stimulatory effect of Ref-1 is, at least partially, achieved by redox regulation of p53. On the other hand, Ref-1 can stimulate DNA binding of wt p53 also by a redox-independent mechanism (Jayaraman *et al.*, 1997), which probably involves modulation of activity of C-terminal domain of p53. HMG-1 belongs to a family of highly conserved chromatin-associated nucleoproteins which bend DNA and facilitate the binding of various transcription factors to their cognate DNA sequence (Onate *et al.*, 1994; Zwilling *et al.*, 1995; Zappavigna *et al.*, 1996). p53 also bends DNA when bound to its cognate sequence (Balagurumoorthy *et al.*, 1995; Nagaich *et al.*, 1997), therefore it is very likely that effect of HMG-1 in stimulation of p53 involves bending of DNA.

3. RESULTS AND DISCUSSION

3.1. Concentration-dependent activation of transcription by p53 (I)

As described in review of literature, p53 can both activate and repress transcription. The p53 binding site in the vicinity of promoter is needed for activation of transcription, while the promoters repressed by p53 usually do not contain p53 binding sites. However, in the case of *Drosophila* Krüppel protein, it has been shown that the same protein can activate or repress the same promoter. The promoter is activated by low concentration of the Krüppel and is repressed when Krüppel is expressed at high concentration (Sauer and Jäckle, 1991). To test whether activation of p53 responsive promoter is similarly dependent on concentration of p53 protein, we cotransfected cells with various amounts of wt p53 expression plasmid and with reporter plasmid containing high-affinity p53 binding site CON in front of the minimal promoter and CAT gene. In Cos7 cells, which contain endogenous wt p53, transfection of low amount of p53 expression plasmid (1–100 ng) lead to slight activation of CAT gene expression, but transfections with higher amounts of wt p53 expression plasmid (1 and 5 μg) caused repression of the promoter far below the basal activity (I, Fig. 2). We also confirmed that the amount of p53 protein in the cell correlates with amount of expression plasmid used in transfections (I, Fig. 1). Next we tested whether the repression p53-responsive promoter depends on the presence of transactivation domain of p53. Although exact mechanism of repression of other promoters by p53 is not known, it has been shown that p53 deficient for transactivation is also unable to repress transcription (Subler *et al.*, 1994). Therefore, we deleted the transactivation domain from p53 and asked whether this truncated protein is able to repress basal transcription from p53-responsive promoter. Again, high amount of expression plasmid (1 and 5 μg), encoding N-terminally truncated p53, caused repression of basal CAT gene transcription from reporter plasmid (I, Fig. 2). This result shows that repression of p53-responsive promoter is independent from transactivation domain and is therefore probably different from the mechanism of repression described previously. We also studied the dose-dependent effects of transactivation by wt p53 in Saos2 cells, which do not contain endogenous p53. Also in this system the moderate amount of wt p53 (10–100 ng of expression plasmid) gave maximum activation of transcription, whereas higher amounts of expression plasmid caused loss of transactivation (I, Fig. 3; II, Fig. 2). We were not able to test the influence of N-terminally truncated p53 in this system, because of very low basal activity of reporter promoter in Saos2 cells.

One possible way to explain the dose-dependent effect of p53 on transcription is to assume that the transfected DNA itself, and not the protein expressed

from it, is responsible for this effect. The plasmid DNA used for transfections contains eukaryotic promoter and is therefore able to bind cellular proteins essential for transcription. This could in turn be the real reason for the transcriptional inhibition described by us. To test the possible side effects of expression plasmid we transfected Saos2 cells with different amounts of wt p53 expression plasmid, keeping total amount of plasmid the same in all transfections. For that we used the same type of expression vector, which contains most of the p53 cDNA sequences, but it also contains 750 bp insertion between ATG codon and p53 coding region, leaving the latter out of frame. Therefore, no p53 protein was expressed, but cDNA sequences were still present. Also in the context of high amount of expression plasmid in the cell, the best activation of transcription by wt p53 was achieved at moderate amounts of the protein (I, Fig. 3). Therefore we concluded that concentration-dependent activation of transcription by p53 is indeed caused by different amounts of p53 protein and not by different amounts of expression plasmid used in transfections.

Taken together, these results show that concentration of p53 can be a factor modulating the level of expression from p53-resposible promoter. Later studies by others in *in vivo* model systems also demonstrated discordance between the transcriptional activity of p53 and its protein level in the cells treated with UV irradiation. Expression of p53 target genes was observed only in the cases, where moderate level of p53 was induced. After induction of high level of p53 protein, activation of p53 target genes (Mdm2 and Waf1) was either significantly delayed or transcription of these genes was not activated at all (Lu *et al.*, 1996; Reinke and Lozano, 1997; Wu and Levine, 1997; Saucedo *et al.*, 1998).

3.2. Oligomerization of p53 is necessary for inhibition of transactivation (II)

We continued the work on concentration-dependent transactivation attempting to determine the structural domains of p53 which are needed for this activity. Our initial data showed that all mutants which oligomerization domain deleted were able to activate transcription and this activity was not inhibited at high protein concentrations. To test more precisely the role of oligomerization in loss of transactivation at high protein concentration, we made deletion mutant Δ324–355, which encodes p53 without the oligomerization domain. We also used another p53 mutant, 1262, which contains four point mutations in the oligomerization domain and has been shown to be monomeric in solution (Stürzbecher *et al.*, 1992). First we confirmed that these mutants are monomeric indeed. For that we transfected cells with appropriate expression constructs, lysed the cells with mild detergent, crosslinked proteins with glutaraldehyde and separated them in the SDS-polyacrylamide gel. After transferring proteins

to nitrocellulose filter, we detected protein complexes by Western blotting with anti-p53 antibodies pAb240 and pAb1801 and identified mono-, di- and tetramers according to molecular weight markers (II, Fig. 1B). Next we cotransfected Saos2 cells with different amounts of expression plasmids together with p53-responsive CAT reporter construct and determined CAT activity 24 hours later. In contrast to wt p53 the monomeric mutants did not show any loss of transactivating activity at high protein concentration (II, Fig. 2). Because different mutants could have different expression levels or half-lives in the cell, we needed to determine whether "high" and "low" concentration of different mutants has the same meaning in every case. We determined relative amounts of p53 protein in every transfection by Western blotting and quantitated protein signals with FluorImager. One half of cells from every transfection was used for CAT assay and the second half was lysed for Western blotting. We detected both p53 and TBP proteins with mixture of monoclonal antibodies on the same blot. 43kD TBP band was easily separable from p53 bands and was used for determination of amount of total protein in particular sample. Next we normalized the signals of p53 bands according to the amount of total protein (TBP band from Western blot) and efficiency of transfection (determined for every series of transfection by β-galactosidase assay). These data showed that the same amount of different expression constructs produced roughly the same amount of different p53 proteins (II, Fig. 3) indicating that all CAT assays were performed in comparable conditions.

It has been shown that oligomerization of p53 is needed for interaction with mdm2 protein (Marston *et al.*, 1995). Therefore it was possible that the loss of transactivating activity of wt p53 could be a result of mdm2-dependent inactivation of p53 and monomeric mutants retained their activity because they were resistant to the mdm2 expression. To evaluate possible role of mdm2 protein in regulation of concentration-dependent transactivation by p53, we used mutant ΔI, which is unable to interact with mdm2 and is resistant to mdm2-dependent inactivation and degradation (Marston *et al.*, 1994; Kubbutat *et al.*, 1997). ΔI mutant behaved similarly to wt protein, indicating that mdm2 is probably not involved in inhibition of transactivation by p53 at high protein concentrations (II, Fig. 2C). We also tested whether changes in specific DNA binding of p53 are responsible for loss of transactivation at high expression level and found that DNA binding activity of p53 correlates with amount of protein in the cells, but not with the transactivating activity of p53 (II, Fig. 4).

Because p53 has to bind proteins of the basal transcription machinery to activate transcription, we propose that at overexpression conditions p53 can titrate out some basal transcription factor(s), amount of which is limited in the cell. This phenomenon is characteristic for many transcriptional activators and is known as squelching (Ptashne, 1988). At optimal concentrations every activator molecule bound to the test promoter is also bound to the target molecule(s) (for example TFIID complex). In excess of the activator most of activa-

tor proteins bound to the test promoter are not bound to the target molecule(s) because of limited amount of the latter. Therefore, activation of the test promoter is inhibited as compared to the optimal concentration of activator. On the other hand, high levels of monomeric p53 mutants did not cause the effect of squelching. Possible explanation here is that monomeric p53 can not interact with transcription machinery at all and therefore is unable to squelch basal transcription factors. If it is so, how can monomeric p53 activate transcription? p53 binding DNA consensus sequence contains two tandem decameric elements, each containing two pentameric inverted repeats. Therefore, the DNA itself can be a factor, which brings four p53 monomers together and p53 could gain the ability to interact with transcription machinery only after binding to the DNA consensus sequence. Increasing concentration of monomeric p53 in the cell could increase probability that more test promoters are bound to multiple p53 monomers, which in turn assures higher transcription efficiency from the promoter.

The second possibility, which should be kept in mind, is that unlike wt protein monomeric p53 is not able to induce apoptosis. Therefore the loss of transactivating activity of wt p53 could be an accompanying effect of the beginning of apoptosis. One could argue that monomeric p53 retains its transactivating properties, but does not induce apoptosis and in the latter case the transactivating activity of wt p53 is not involved in induction of apoptosis. However, we were not able to detect differences in viability of cells 24 hours after transfection, when CAT assays were done.

3.3. Mutant p53 can inhibit activities of p53 without heterooligomerization (III)

In human tumors p53 protein has been often mutated. Because it is expressed from both alleles, the overall content of intracellular p53 can be either wild-type (wt), mixture of wt and mutant p53 or mutant p53 only (when both alleles are mutated or one allele is deleted). In the case of coexpression of mutant and wt p53 it has been shown that mutant p53 can drive wt p53 to mutant conformation (Milner and Medcalf, 1991) and the transactivation function of wt p53 is inhibited by mutant p53 (Kern *et al.*, 1992). It is generally believed that the mechanism behind this effect is that mutant p53 proteins form heterooligomers with wt proteins, which are less active in transcriptional activation than homooligomers of the wt protein. If this is the case, the monomeric p53 would be insensitive to mutant protein, because heterooligomers cannot form. To test this we carried out series of transfections in Saos2 cells, where monomeric mutant Δ324–355 was used as activator of transcription and it was co-expressed with three different cancer-associated point mutants of p53. All transfections con-

tained also two reporter plasmids. One of them had high-affinity p53 binding site CON in front of minimal promoter and CAT gene, which was used for measuring of p53-dependent transcription. Another reporter contained CMV promoter in front of β-galactosidase gene, which indicated the activity of p53-independent transcription. To our surprise, all point mutants tested clearly inhibited p53-dependent transcription when compared to co-expression with empty expression plasmid (III, Fig. 2A). p53-independent transcription was not changed by mutants (III, Fig. 4). To get more insight into the nature of this inhibition we deleted N-terminal transactivation and/or C-terminal regulatory domain(s) of the Trp248 mutant p53 protein (III, Fig. 1) and tested them in our assay. When either N- or C-terminus was deleted, the mutant could still inhibit the transcriptional activity of the Δ324–355. However, when both termini were absent, the mutant was not able to repress the Δ324–355 activity any more (III, Fig. 2B). Reporter plasmid used in these experiments contained synthetic p53 binding site CON in front of the adenovirus E2 promoter. This binding site binds p53 very efficiently, but does not occur in natural promoters. To test our finding in more physiological context, we also performed the same kind of experiments using a reporter plasmid containing 2.4 kb fragment from human Waf1 promoter in front of the CAT gene and the result obtained was essentially the same: only the mutant with both termini deleted was unable to inhibit transactivation by monomeric p53 (III, Fig. 3).

In order to clarify the relevance of our results in more biological assay, we tested the influence of point mutants to the growth suppression activity of Δ324–355. It has been reported that monomeric p53 expresses clear growth suppressing activity, although slightly less than the wt protein (Shaulian *et al.*, 1993; Thomas *et al.*, 1996). In our hand the wt p53 inhibited colony formation almost entirely and Δ324–355 reduced the number of colonies approximately 50% as compared to the transfections with the resistance marker only. When either Trp248, its N-terminal or C-terminal deletion mutants were added to the transfection mixture, they inhibited the growth suppressing activity of the monomer (III, Fig. 8). The Trp248 mutant with both termini deleted was in itself able to reduce the colony number therefore, its effect to the activity of the monomer could not be examined.

Next we tested, whether mutant proteins change the expression level or localization of monomeric mutant Δ324–355. Because most of the deletion mutants used had different electrophoretic mobilities in SDS-gel, we were able to control the expression level of our proteins by Western blotting. We did not detect any change of expression of Δ324–355 when different mutants were co-expressed (III, Fig. 5). We also tested whether Δ324–355 was located in the nucleus during coexpression of different mutants. For this purpose we coexpressed deletion mutants of Trp248 together with Δ324–355 protein and determined localization of the latter by immunofluorescent staining with antibodies, epitopes of which were lacking in Trp248 mutants. In all cases Δ324–355 was

located in the nucleus (III, Fig. 6). We also confirmed that Δ324–355 did not form oligomers with Trp248 deletion proteins. Cells cotransfected with Δ324–355 and deletion mutants of Trp248 were lysed, crosslinked with glutaraldehyde, divided between two equally loaded gels, electrophoresed and blotted to nitrocellulose. By using Western blotting with different primary antibodies we were able to show that in the case of coexpression with point mutants the Δ324–355 remains monomeric in solution (III, Fig. 7).

Taken together, we have shown that in coexpression conditions mutant p53 can inhibit the activity of functional p53 without heterooligomerization and this effect is not caused by improper expression or localization of the latter. This inhibition was not extended to transcription from p53-independent promoter. We propose that the mutant form of p53, when expressed at high level, may deplete cells from cofactors necessary for p53 to activate transcription, but not needed for transcription in general. The transactivating function of p53 can be modified by different factors. The c-Abl and WT-1 are cellular proteins binding wt p53 and activating its transactivation function (Maheswaran *et al.*, 1993; Goga *et al.*, 1995; Maheswaran *et al.*, 1995). Recently, new activators of p53 were identified, including Ref-1 (Jayaraman *et al.*, 1997), HMG-1 (Jayaraman *et al.*, 1998), $p33^{ING1}$ (Garkavtsev *et al.*, 1998), BRCA1 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998) and 14–3–3 (Waterman *et al.*, 1998). It is not known, whether they can interact with point mutants of p53 or with monomeric p53. Therefore we have no clues to speculate, could one of these activators be depleted from cells by mutant p53 in our experiments.

Two explanations can be proposed to the fact that Trp248 mutant, truncated at both termini, cannot inhibit Δ324–355 mediated transactivation. First, the deletions from both ends of the protein may affect the overall conformation of the molecule making it unable to bind the specific cofactor. The other explanation comes from a hypothesis according to which N- and C-termini are close to each other in 3-dimensional structure. They could generate a binding site for this proposed cofactor and only the deletions of both of them abolish the binding of the cofactor. Alternatively, wt p53 may need a specific modification for its activity rather than binding to a specific cofactor. If p53 needs to be phosphorylated in order to activate transcription, the inhibitory effect of point mutants to Δ324–355 mediated transcription may relay on the saturation of the modifying enzymes. Following this line two or more modifications are needed for transactivation by p53 — at least one for N- and the other for C-terminus. If even one of these possible modifying enzymes is depleted by mutant p53, the functional p53 cannot activate transcription. Only in the case of Trp248, truncated from both termini, the Δ324–355 retains its activity, because the former is not able to bind modifying enzyme(s) needed for modification of p53.

4. CONCLUSIONS

The present study can be summarized as follows:

1. We have shown that the best activation of p53-responsible promoter is achieved by optimal concentration of p53 protein. p53 loses its transactivating activity at high protein concentrations.

2. Inhibition of transactivation at high protein concentration is not the result of loss of sequence-specific DNA binding activity of p53 and is not caused by mdm2-dependent inactivation of p53.

3. Oligomerization of p53 is involved in inhibition of transactivation at high protein concentrations.

4. Cancer-associated point mutants of p53 can inhibit activities of functional p53 without heterooligomerization.

5. Both N- and C-termini of mutant p53 are involved in the inhibition of activities of monomeric p53. Only the mutant with both termini deleted is inactive for this inhibition.

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KASVAJATE SUPRESSORVALGU p53 OMADUSED TRANSKRIPTSIOONI AKTIVAATORINA

Kokkuvõte

p53 on rakuline valk, mille peamiseks funktsiooniks on rakkude normaalse kasvu ja jagunemise kontrollimine. Koos funktsionaalse p53 kadumisega rakus suureneb kasvaja arenemise tõenäosus, mida kinnitab ka fakt, et üle 50%-s kasvajatest on p53 geen muteerunud. Mutatsioonid on kõige sagedasemad p53 DNA-d siduvas domäänis ning jagunevad valdavalt kahte tüüpi: mutatsioonid, mis muudavad otseselt DNA-ga kontakteeruvat aminohappejääki, ning mutatsioonid, mis lõhuvad domääni üldise struktuuri. Et aktiivne p53 moodustub nelja monomeeri tetramerisatsioonil, siis heterosügootses rakus moodustub mutantse ja normaalse p53 kompleks. Selline kompleks on kas ainult osaliselt funktsionaalne või täiesti inaktiivne. Sellega seletub ka mutantse p53 võime inaktiveerida normaalse valgu funktsioone. p53-l on mitmeid aktiivsusi, millest seni on kõige paremini uuritud tema võimet mõjutada erinevate geenide transkriptsiooni. p53 aktiveerib mitmeid geene, kuid on ka paljude repressoriks. Transkriptsiooni aktiveerimiseks peab p53 seonduma basaalsete transkriptsioonifaktoritega ning järjestusspetsiifiliselt DNA-ga. Käesolev doktoritöö põhineb kolmel uurimusel, millest kahes me selgitasime p53 poolt aktiveeritava transkriptsiooni sõltuvust p53 kontsentratsioonist rakkudes ning kolmandas näitasime, et normaalse p53 funktsiooni häirib mutantne valk isegi siis, kui need kompleksi ei moodusta. Lühidalt olid tulemused järgmised.

p53 aktiveerib transkriptsiooni optimaalse kontsentratsiooni juures. Kui p53 valku on rakus üle optimaalse hulga, siis p53-st sõltuv transkriptsiooni aktivatsioon väheneb oluliselt. Me näitasime, et selline aktiivsuse langus pole tingitud p53 DNA-d siduva võime vähenemisest ning tõenäoliselt samuti mitte p53 negatiivse regulaatorvalgu mdm2 ekspressioonist. p53 deletsioonanalüüsil leidsime, et sellise inhibitsiooni tekkeks on vajalik p53 oligomeriseerumine: monomeersed mutandid aktiveerisid transkriptsiooni ka siis, kui neid ekspresseerida kõrgel kontsentratsioonil.

Ekspresseerides samaaegselt koos nii mutantset kui ka normaalset p53 valku, moodustavad need funktsionaalselt inaktiivse heterooligomeeri. Et vältida heterooligomeeride teket, kasutasime oma katsetes transkriptsiooni aktiveerimisel monomeerset p53. Sellise valgu funktsiooni ei peaks häirima mutantne p53, sest heterooligomeere tekkida ei saa. Selgus aga, et mutantne p53 on võimeline inaktiveerima ka monomeerse valgu funktsioone. Mutandi inhibeeriv mõju kadus, kui sellest olid deleteeritud nii transaktiveeriv kui ka regulatoorne domään. Kumbki deletsioon üksinda ei kaotanud mutantse p53 võimet inhibeerida monomeerse p53 transaktiveerivat aktiivsust. Samas ei

mõjutanud mutantse p53 (ega tema deletsioonimutantide) ekspressioon p53-st mittesõltuva transkriptsiooni toimumist. Saadud andmed lubavad oletada, et p53 vajab oma aktiivsuse saavutamiseks mingeid rakulisi kofaktoreid, mille hulk on limiteeritud ning mutantne p53 võib need lihtsalt välja tiitrida.

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I

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Protein p53 modulates transcription from a promoter containing its binding site in a concentration-dependent manner

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Tumor suppressor protein p53 binds to DNA in a sequence-specific manner and activates transcription from promoters near its binding site. It is also known to repress promoters lacking the p53-binding site. In this study, we demonstrate that p53 can act as a transcriptional activator or repressor *in vivo* using the same reporter with the DNA-binding site CON and these effects depend on the amount of p53 expressed. Both in Saos2 and Cos7 cells, lower concentrations of p53 lead to activation and higher concentrations lead to repression of the model promoter containing the consensus p53-binding site CON. The N-terminal part of p53 is necessary for the transcriptional activation. It is not needed, however, for the repression of the same promoter, indicating that different domains of p53 are involved in activation and repression.

Keywords: oncoprotein; p53; transcription; concentration dependence; chloramphenicol-acetyltransferase assay.

The human tumor suppressor protein p53 is able to modulate RNA transcription both *vivo* and *vitro* (Agoff et al., 1993; Barak et al., 1993; Chin et al., 1992; Deb et al., 1992, 1994; El-Deiry et al., 1993; Jackson et al., 1993; Juven et al., 1993; Kastan et al., 1992; Mack et al., 1993; Okamoto and Beach, 1994; Ragimov et al., 1993; Subler et al., 1992, 1994; Zambetti et al., 1992). Wild-type p53 binds to double-stranded DNA homologous or identical to the consensus sequence 5′-RRRC(A/T)(A/T)GYYYN$_{0-13}$RRRC(A/T)(A/T)GYYY-3′ in a sequence-specific manner (El-Deiry et al., 1992; Funk et al., 1992). It has been shown that wild-type p53 can transactivate the promoters containing its DNA-binding site due to interaction of the p53 N-terminal region with the TATA-box-binding protein (TBP; Martin et al., 1993; Subler et al., 1994; Truant et al., 1993; Unger et al., 1993). According to the current model for its function in cell growth inhibition, p53 activates transcription of the *WAF1 (CIP1)* gene, the product of which in turn regulates G1 cyclin-dependent protein kinases and proliferating cell nuclear antigen (El-Deiry et al., 1993; Xiong et al., 1993; Harper et al., 1993; Li et al., 1994).

In contrast, p53 has been shown to repress several viral and cellular promoters containing no known p53-binding sites (Agoff et al., 1993; Deb et al., 1992; Jackson et al., 1993; Mack et al., 1993; Ragimov et al., 1993; Subler et al., 1992, 1994). This effect has been explained as the 'squelching' of basal transcription factors TBP and transcription factor IID (TFIID) by interaction of N-terminal amino acids of p53. Although both TATA-box-mediated and pyrimidine-rich-initiator-element-mediated transcription need TBP for initiation, only the former is susceptible to repression by wild-type p53 (Mack et al., 1993). This indicates that other mechanisms than 'squelching' of transcription factors can be additionally involved in transcriptional repression by the p53 protein.

Mutations in the p53 gene are the most common genetic alterations in human tumors (Hollstein et al., 1991; Nigro et al., 1989). While wild-type p53 protein is able to suppress cell growth and transformation, the tumor-derived mutants have lost these properties and can support transformation of cells (Jenkins et al., 1985; Hinds et al., 1989). Recently, it was shown that most of the p53 mutations found in tumors occur at amino acids directly in contact with DNA or at residues stabilizing the conformation of the DNA-binding domain (Cho et al., 1994). In both cases, p53 is not able to bind its specific DNA target, which leads to its inability to activate the genes responsible for cell cycle control (i.e. *WAF1/CIP1*). However, the p53 gene is not mutated in all cases in any particular type of human tumor. Even in these types of tumors, which most often contain mutations in the p53 gene, the p53 gene is wild-type in at least one third of cases (Hollstein et al., 1994). Clearly, mutations in p53 are not the only reason for loss of cell proliferation control in these cells.

In this study, we investigated the effects of p53 concentration on its transcription-modulating activities *in vivo*. We were interested in how the concentration of p53 protein expressed in cells modulates its ability to activate/repress RNA transcription from a reporter plasmid containing the chloramphenicol acetyltransferase gene under the control of the adenoviral E2 promoter and the consensus p53-binding site CON (Funk et al., 1992). We demonstrate that transcription from the reporter plasmid can be activated or repressed by p53 *in vivo* and these effects depend on the amount of p53 expressed in cells. Lower concentrations of p53 lead to the activation of promoter in the cells lacking endogenous p53 (Saos2) and additional activation in the cells having high levels of endogenous p53 (Cos7). Higher concentrations of p53 lead to the repression of the same promoter in Cos7 cells and loss of activation in Saos2 cells. Truncated p53 protein without the 39 N-terminal amino acids essential for transactivation and TBP binding has lost its transactivating properties, but

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Abbreviations. CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; IMEM, Iscove's modified Eagle's medium; TBP, TATA-box-binding protein.

Enzyme. Chloramphenicol acetyltransferase (EC 2.3.1.28).

is still able to repress transcription. We also demonstrate that the p53 concentration *per se*, in addition to mutations in its amino acid sequence, can be a factor modulating p53-dependent transcription, which could lead to the loss of cell cycle control in tumors.

MATERIALS AND METHODS

Plasmid constructs. Expression plasmid pCG-Hwt contains human wild-type p53 cDNA downstream of the cytomegalovirus immediate early promoter and mRNA leader sequence of the herpes simplex virus (HSV) thymidine kinase gene. pCG-dl39 was created by PCR and encodes the mutant p53 protein with deletion of the first 39 amino acids. Expression, appropriate molecular mass, and nuclear localization of proteins were checked by Western blotting and immunofluorescence staining. Plasmid pCG-RN was created by inserting a 750-bp bovine papillomavirus E2 sequence into pCG-Hwt. This insert replaces DNA sequences coding for amino acids 1–58 of p53 and causes frameshift of p53 sequences.

The E2-100 CAT reporter plasmid was provided by Dr J. Jenkins and was described earlier (Tarunina and Jenkins, 1993). It contains the p53 consensus DNA-binding site CON (Funk et al., 1992) and modified adenovirus E2 promoter in front of the chloramphenicol acetyltransferase (CAT) gene.

Cell culture and transfection. Cos7 and Saos2 cells were cultured in Iscove's modified Eagle's medium (IMEM) supplemented with 10% fetal calf serum and 100 U/ml penicillin and streptomycin. Cells were maintained at 37°C in humidified 5% CO_2 atmosphere. Cells were transfected by electroporation as follows: $5-10\times10^6$ cells were collected from 70% confluent cell dishes and suspended in IMEM containing 5 mM Na-Bes [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]. 50 μg sonicated salmon sperm DNA, and expression and reporter plasmids were added to 250 μl cell suspension and the cells were pulsed (1 mF and 190 V for Cos7 cells, 1 mF and 210 V for Saos2 cells) using an Invitogene electroporator. 100 ng or 2 μg E2-100 CAT reporter plasmid was used for transfection of Cos7 or Saos2 cells, respectively. After electroporation, cells were resuspended in IMEM and transferred to growth conditions on 10-cm-diameter cell culture dishes.

Cell viability was determined 24 h after transfection by trypan blue exclusion. No correlation between cell viability and the amount of transfected plasmids was found (7–10% dead cells in all cases).

Western blotting. Cells were lysed 24 h after transfection in 200 μl SDS loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 3 min. 10% of the lysate was separated by SDS/PAGE in a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose filter and detected with a mixture of monoclonal antibodies pAb421 (Harlow et al., 1981), pAb1801 (Banks et al., 1986), and pAb240 (Gannon et al., 1990). Rabbit anti-mouse IgG secondary antibody and ^{35}S-labeled protein A were used for quantification of p53 protein. Quantitation of data was performed with a PhosphorImager using the ImageQuant software (Molecular Dynamics).

CAT assay. Cells were collected 24 h after transfection. The lysis of cells and CAT assays were performed by standard methods (Sambrook et al., 1989). One third of the Cos7 cell lysate and one half of the Saos2 cell lysate were used in the CAT assay. Enzyme reactions with D-threo(dichloro-[1-^{14}C]acetyl)chloramphenicol and acetyl-coenzyme A were incubated at 37°C for 1 h (Cos7 lysates) or 3 h (Saos2 lysates). Quantitation of data was performed using a PhosphorImager.

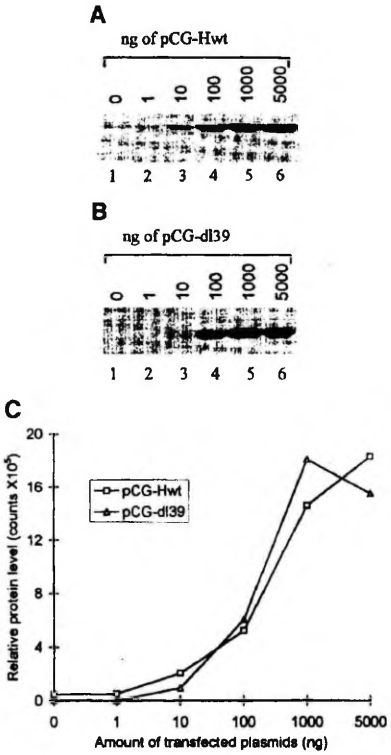

Fig. 1. Western blot of cell lysates transfected with different amounts of expression plasmids. 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 (lane 4), 1000 (lane 5) or 5000 (lane 6) ng plasmid pCG-Hwt (A) or pCG-dl39 (B) were transfected into Cos7 cells. The filter was probed with anti-p53 monoclonal antibodies, rabbit anti-mouse antibodies, and ^{35}S-labeled protein A. (C) Quantitation of data from A (□) and B (△) with PhosphorImager.

RESULTS AND DISCUSSION

Several authors have shown that wild-type p53 can activate transcription from promoters containing the consensus DNA-binding sequence (Barak et al., 1993; Deb et al., 1994; El-Deiry et al., 1993; Kastan et al., 1992; Juven et al., 1993; Zambetti et al., 1992) and can repress promoters without such a site (Agoff et al., 1993; Jackson et al., 1993; Mack et al., 1993; Ragimov et al., 1993; Subler et al., 1992). The interpretation for p53-dependent repression of transcription is that p53 binds to TBP and drives it to promoters near the p53-binding site, therefore 'squelching' basic transcription factors from promoters not containing the p53-binding site. Usually, the amount of p53 expression plasmid used in these experiments was high (5–10 μg/transfection).

Cos7 cells were transfected with different amounts of the p53 expression construct pCG-Hwt, containing human p53 cDNA under the control of the cytomegalovirus immediate early promoter and the HSV thymidine kinase mRNA leader sequence. Western blotting of the cell lysates was used to demonstrate that in the range 1 ng to 5 μg expression plasmid used for transfection, the amount of protein expressed increased (Fig. 1A, lanes 2–6, and Fig. 1C).

We also constructed a deletion mutant of human p53, pCG-dl39, which encodes the protein lacking the N-terminal 39 amino acids. These sequences have been shown to be essential for transcriptional *trans*-activating properties of p53 and contain the binding site for TBP (Liu et al., 1993; Unger et al., 1993). Amino acid residues Leu22 and Trp23 have been shown to be the most critical residues for the transactivation activity (Lin et al., 1994). Transfection of Cos7 cells with increasing amounts of this construct also leads to an increase in the level of protein expressed (Fig. 1B, lanes 2–6, and Fig. 1C). The band observed in lane 1 of Fig. 1A and the upper band in Fig. 1B correspond to endogenous p53 from Cos7 cells.

Immunofluorescent staining of the transfected cells showed that the efficiency of transformation (about 30% cells transfected) did not depend on the amount of expression plasmid used (data not shown). Therefore, the increase in the level of protein shown in Fig. 1 is a real increase of the intracellular protein concentration. Similar results were obtained with human Saos2 cells lacking endogenous p53 sequences (Masuda et al., 1987; data not shown). The level of expression of p53 in these cells was lower because of lack of amplification of the expression plasmid in Saos2 cells.

We tested whether p53 expressed from transfected plasmid could change the transactivation of the promoter by endogenous p53 protein in Cos7 cells (Fig. 2). These cells express endogenous p53 protein at a level that is sufficient to activate the exogenous E2 promoter containing the specific p53-binding site (Fig. 2A, lane b). The additional expression of a small amount of exogenous wild-type p53 causes a slight additional activation of transcription (Fig. 2A, lanes 1 and 2). The expression of high amounts of p53 protein in Cos7 cells causes repression of transcription far below the basal level (Fig. 2A, lanes 4 and 5, and Fig. 2C). This experiment shows that high concentrations of p53 are able to reverse the transactivating effects caused by endogenous monkey p53 protein in Cos7 cells.

It has been shown recently that both transactivating and oligomerization domains of the p53 protein are needed for the repression of human cytomegalovirus immediate early promoter containing no p53-binding sequences (Subler et al., 1994). We tested whether the transactivating domain of p53 is also needed for the transcriptional repression of the promoter with the p53-binding site described here. For this purpose, we constructed the expression vector pCG-dl39, encoding human p53 protein without the N-terminal 39 amino acids. These amino acids are essential for the transcriptional transactivation activity of p53 and binding of the basal transcription factor TBP (Liu et al., 1993; Unger et al., 1993). The truncated protein is able to repress the basal level of reporter transcription caused by endogenous p53 protein in Cos7 cells (Fig. 2B, lanes 4 and 5, and Fig. 2C). PhosphorImager quantitation of three independent CAT assays (both with pCG-Hwt and pCG-dl39) is shown in Fig. 2C. These results show that the transcriptional repression of promoters by high concentration of the p53 protein is independent from the transcription-activation domain of the p53 protein.

We also studied the dose-dependent effects of human p53 protein on CAT gene transcription in Saos2 cells. Fig. 3A shows that the transactivating effect of p53 in Saos2 cells depends on the concentration of protein in the cells. The activating effect appears at a relatively low level of the expression construct used (Fig. 3A, lanes 3 and 4). Higher amounts of expression plasmid used lead to the loss of activation of transcription in the same system (Fig. 3A, lanes 5–7).

One possible way to explain the dose-dependent effect of p53 on transcription described by us is to assume that the transfected DNA itself, and not the protein expressed from it, is

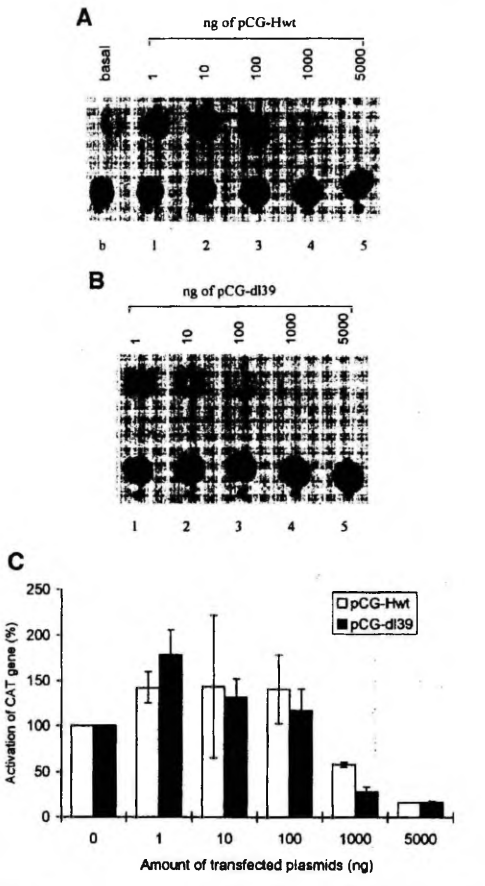

Fig. 2. Dose-dependent effect of p53 protein on CAT gene transcription in Cos7 cells. Cos7 cells were transfected with expression plasmids pCG-Hwt (A) or pCG-dl39 (B) and 100 ng reporter plasmid E2-100 CAT. 1 (lane 1), 10 (lane 2), 100 (lane 3), 1000 (lane 4) or 5000 (lane 5) ng expression plasmid were used. Lane b in A shows the basal CAT activity of Cos7 cells transfected with reporter plasmid only. (C) Quantitation of data from three independent transfection experiments with pCG-Hwt (empty columns) and pCG-dl39 (dotted columns).

responsible for this effect. The plasmid DNA used for transfections contains the eukaryotic promoter and is therefore able to bind cellular proteins essential for transcription; this could in turn be the real reason of the described transcriptional inhibition. However, we have shown that this is not the case. Two sets of experiments were performed. We transfected Saos2 cells with different amounts of wild-type p53 expression plasmid, keeping the total amount of plasmid (5 μg, in addition to 2 μg reporter plasmid E2-100 CON) the same in all transfections. For this we used a plasmid pCG-RN containing most of the p53 cDNA sequences, but which did not express them as protein because of a 750-bp DNA cloned between ATG and p53 leaving the latter out of frame. Therefore, no p53 protein sequences were expressed from this plasmid, but the DNA sequences were still present. As shown in Fig. 3B and C, the effect of p53 protein concentration evident in Fig. 2A and Fig. 3A was also detecta-

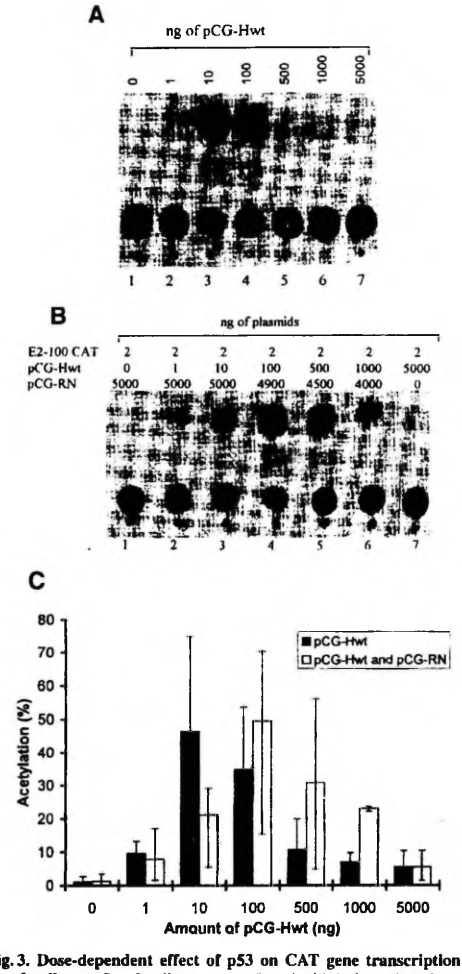

Fig. 3. Dose-dependent effect of p53 on CAT gene transcription in Saos2 cells. (A) Saos2 cells were transfected with 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 (lane 4), 500 (lane 5), 1000 (lane 6) or 5000 (lane 7) ng plasmid pCG-Hwt and 2 μg reporter plasmid E2-100 CAT. (B) The same amounts of expression and reporter plasmids as in A were used and also 5000 (lanes 1–3), 4900 (lane 4), 4500 (lane 5), 4000 (lane 6) or 0 (lane 7) ng plasmid pCG-RN was added to transfections to keep constant the overall amount of transfected DNA. (C) Quantitation of data from three independent experiments, where Saos2 cells were transfected with pCG-Hwt in the presence (empty columns) and absence (dotted columns) of plasmid pCG-RN. In the former case, the overall amount of transfected DNA was kept the same (5 μg), changing the ratio of plasmids pCG-Hwt and pCG-RN in different transfections.

ble in this experiment. Moderate amounts (10–100 ng) of p53 expression plasmid lead to promoter activation, whereas higher amounts cause loss of this activity. In addition, we transfected Cos7 cells with plasmid pCG-RN and did not detect transcriptional repression effects similar to those described in Fig. 2 (data not shown). Therefore, we believe that the dose-dependent transcriptional effects of p53 protein described in this study are indeed caused by the protein and not nucleic acid sequences.

The results in this study show that the intracellular concentration of p53 *per se* can be a factor modulating the level of expression from promoters regulated by the p53 protein. In addition to the p53 mutations found in human tumors, which are most likely the reason for the loss of cell growth control in human tumors, one can also predict that overexpression of wild-type p53 protein in cells can lead to the abregulation of p53-dependent promoters and loss of cell-growth control. It is possible, therefore, that several human tumors containing the wild-type p53 sequence still develop because of defects in p53-dependent pathways.

There are also other proteins in eukaryotic cells that have been shown to act as concentration-dependent regulators of transcription. Low amounts of Krüppel protein led to transcriptional activation (Sauer and Jäckle, 1991), whereas high levels of Krüppel resulted in gene repression (Sauer and Jäckle, 1991; Licht et al., 1990); both effects were mediated by the same 11-bp Krüppel-binding site. Krüppel lacking either the 64 C-terminal amino acids or the 116 N-terminal amino acids could only activate or repress gene transcription showing that different parts of the Krüppel protein are essential for gene activation and repression (Sauer and Jäckle, 1991). Similar evidence for the dual function of Hunchback protein have been published, where concentration-dependent activation or repression are mediated by the same *cis*-acting element (Zuo et al., 1991).

It has been shown that p53 can act as a transactivator of transcription from some promoters and as a repressor from others (Agoff et al., 1993; Barak et al., 1993; Chin et al., 1992; Deb et al., 1992, 1994; El-Deiry et al., 1993; Jackson et al., 1993; Juven et al., 1993; Kastan et al., 1992; Mack et al., 1993; Okamoto and Beach, 1994; Ragimov et al., 1993; Subler et al., 1992, 1994; Zambetti et al., 1992). Each activity in these cases involves a distinct set of *cis*-acting sequences. In this paper, we have shown that p53 can activate or repress transcription from the same binding site in a concentration-dependent manner. The ability of p53 protein to effect the activation and repression of transcription by its interaction with a single binding site provides a new aspect for gene regulation in p53-dependent pathways using concentration-dependent regulatory mechanisms.

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SHORT REPORT

Oligomerization of p53 is necessary to inhibit its transcriptional transactivation property at high protein concentration

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We have previously shown that transactivation by tumor suppressor protein p53 can be inhibited *in vivo* at elevated protein concentrations. In this study we characterize the structural requirements of this function. We show that oligomerization domain of p53 is involved in loss of transactivation at high protein concentrations: mutants not able to oligomerize are neither able to suppress transactivation, although these transactivating properties can be untouched.

Keywords: p53; oligomerization; transactivation

Tumor suppressor protein p53 is involved in regulation of transcription activating the promoters located near to its DNA binding sites. Number of such genes are identified. a part of them is clearly involved in regulation of cell proliferation (Barak *et al.*, 1993; Buckbinder *et al.*, 1995; El-Deiry *et al.*, 1993; Kastan *et al.*, 1992; Miyashita and Reed, 1995; Okamoto and Beach, 1994; Owen-Shaub *et al.*, 1995). p53 also represses many viral and cellular promoters (Deb *et al.*, 1992, 1994; Desaintes *et al.*, 1995; Jackson *et al.*, 1993; Subler *et al.*, 1992, 1994). Exact mechanism of transrepression is obscure.

For transactivation, p53 must interact with the basal transcription machinery and bind sequence-specifically to DNA. The corresponding DNA sequence has been characterized having consensus 5'-RRRC(A/T)(A/T)GYYYN$_{0-13}$RRRC(A/T)(A/T)GYYY-3' (El-Deiry *et al.*, 1992; Funk, *et al.*, 1992).

Several functional domains on p53 amino acid chain have been characterized. The DNA binding domain of p53 is mapped between amino acids 102 and 290 (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). The N-terminal domain of p53 (amino acids 1 to 42) is needed for transactivation and it interacts with basal transcription factors. p53 protein is able to form tetramers *in vivo*, containing the oligomerization domain mapped between amino acids 323 and 355 (Clore *et al.*, 1994; Wang *et al.*, 1994). Monomeric p53 retains transactivating ability (Crook *et al.*, 1994; Pellegata *et al.*, 1995; Sang *et al.*, 1994; Tarunina and Jenkins, 1993; Wang *et al.*, 1993), but is incompetent for transrepression function (Crook *et al.*, 1994; Pellegata *et al.*, 1995; Sang *et al.*, 1994).

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We have previously shown that at elevated expression conditions the transactivating property of wt p53 is strongly inhibited. In model systems, lower concentrations of p53 lead to activation of a promoter containing the consensus DNA binding site and higher concentrations to loss of this activity (Kristjuhan and Maimets, 1995). The same phenomenon was also observed in cell lines where increase of endogenous p53 was induced with U.V. radiation (Lu *et al.*, 1996). In present study we show that oligomerization is necessary for 'self-inhibition' of transactivation by p53.

To study the transactivation properties of p53 we used a set of p53 mutants in CAT assays (Figure 1a). Mutant ΔI has the first evolutionary conserved domain of p53 deleted (Soussi *et al.*, 1990), but it behaves similarly to wt protein in transactivation, transrepression and growth suppression assays (Crook *et al.*, 1994). 1262 contains point mutations in C-terminus of protein disrupting p53 tetramers into monomers (Stürzbecher *et al.*, 1992; Tarunina and Jenkins, 1993). Mutant Δ324–355 has the oligomerization domain of p53 deleted. We controlled expression of proteins from our constructs in Saos2 cells, which do not contain endogenous p53 protein. All p53 proteins were expressed at comparable levels and immunofluorescent staining of transfected cells showed that all p53 proteins were located in the nucleus (data not shown).

The oligomerization domain of p53 is located between amino acids 323 and 355 (Clore *et al.*, 1994; Wang *et al.*, 1994). Mutants Δ324–355 and 1262 were expected to be monomers as Δ324–355 has oligomerization domain deleted and 1262 contains four point mutations which abolish oligomerization of p53 (Stürzbecher *et al.*, 1992). We determined the ability of p53 proteins to form homooligomers *in vivo*. Saos2 cells were transfected with p53 expression plasmids and lysed 24 h later in IP buffer. Equal amounts of lysate were crosslinked with different concentrations of glutaraldehyde and p53 complexes were detected by Western blotting. As expected, mutant ΔI and wt protein can efficiently form homodi- and tetramers *in vivo*, while Δ324–355 and 1262 form monomers only (Figure 1b).

To test the ability of p53 mutants to activate transcription at different concentrations, we cotransfected Saos2 cells with 1 μg of pBS-CON-CAT reporter plasmid and increasing amounts of p53 expression plasmids. Cells were collected and lysed 24 h after transfection. Before CAT reaction all lysates were normalized according to total amount of protein and efficiency of transfection, thus every reaction contained the same amount of cells positive for p53 expression.

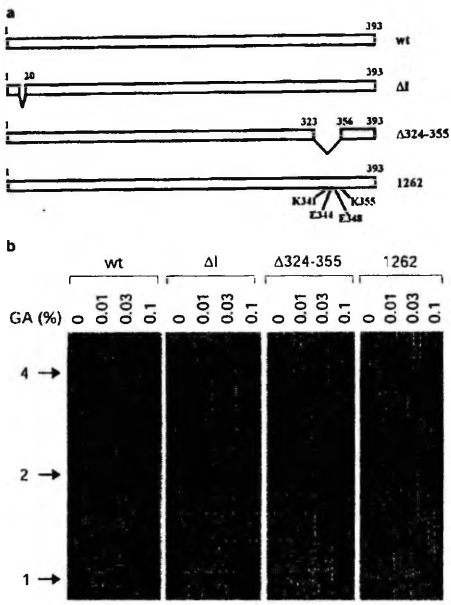

Figure 1 Oligomerization of p53 proteins. (a) Schematic representation of p53 proteins used in this study. All p53 cDNA-s were cloned between *Xba*I and *Bgl*II sites of eukaryotic expression vector pCG (Tanaka and Herr, 1990). ΔI encodes p53 with deletion in the first conserved domain of p53 (amino acids 13–19). For creating Δ324–355, two regions of wt p53 cDNA were amplified: one between codons 1 and 323 and another between codons 356 and 393. Resulting cDNA-s were linked in frame to each other from *Pst*I site designed into PCR primers. Half of *Pst*I site (CAG) encodes an extra amino acid (Glu) between these two regions. 1262 contains four point mutations in oligomerization domain and is described previously (Tarunina and Jenkins, 1993). (b) Oligomerization of p53 mutants. Cells transfected with 2 μg of p53 expression plasmids, were lysed in IP buffer (500 mM NaCl, 50 mM Hepes pH 7.5, 1 mM EDTA, 0.5% NP40) and crosslinked with different concentrations of glutaraldehyde. Protein complexes were separated in 6% SDS–PAAG, transferred to nitrocellulose filter and blotted with mixture of antibodies pAb240 and pAb1801. Concentration of glutaraldehyde (GA) is indicated on top of lanes. Bands corresponding to mono-, di- and tetramers are indicated on left side (1, 2 and 4, respectively)

As expected, the highest activation of reporter gene by wt p53 was observed in transfections with relatively low concentration of expression plasmid (Figure 2a), while increasing amounts of expression plasmid caused decrease in CAT activity.

MDM2 protein can inactivate transactivation capability of p53 interacting with transactivation domain of p53 and directing its degradation (Haupt *et al*., 1997; Kubbutat *et al*., 1997). Transcription of MDM2 gene is activated in p53 dependent manner (Barak *et al*., 1993). Therefore it is possible that high expression of p53 protein causes accumulation of MDM2 protein, which in turn inactivates p53. To avoid effects of MDM2 protein we used the p53 ΔI mutant, which has MDM2 binding domain deleted and is resistant to MDM2-dependent inactivation and degradation (Kubbutat *et al*., 1997). Deletion of the first evolutionarily conserved domain of p53 caused decrease in the absolute level of reporter activation, but overall pattern of transactivation was the same: at higher amounts of expression plasmid the CAT activity was inhibited (Figure 2b). At the same time activations of reporter gene by monomeric p53 mutants Δ324–355 and 1262 were not inhibited at higher concentrations of expression plasmid and CAT activity reached to plateau (Figure 2c and d). The loss of self-inhibition is likely to intrinsic property of monomeric p53 as results with both monomeric p53 mutants (deletion and point mutant) were similar. As a negative control we used the His175 point mutant, which did not activate reporter gene at any concentration (data not shown).

The expression vector pCG contains strong eukaryotic promoter (cytomegalovirus immediate early promoter) able to bind cellular proteins essential for transcription. Therefore one could argue that high amount of pCG vector itself can be responsible for repression of transcription from other promoters. However, since all p53 proteins were expressed from the same type of vector, the results from CAT assays with Δ324–355 and 1262 proteins show that decrease of CAT activity by wt and ΔI proteins is not caused by high level of pCG plasmid itself. While wt and ΔI proteins can activate reporter gene already at very low concentrations (1 and 10 ng of expression plasmid), monomeric p53 mutants Δ324–355 and 1262 had no detectable activation at less than 100 ng of expression plasmid used. It can reflect the decreased DNA binding activity of monomeric proteins.

To be sure that all p53 mutants were expressed at comparable levels and cover the same ranges of concentrations we defined the relative amount of p53 in all lysates used for CAT assays. Transfected cells were collected 24 h after transfection and divided between two tubes. Half of cells was lysed for CAT assay, the second half was lysed for Western blotting directly in SDS loading buffer. All protein lysates were separated in SDS–PAGE at the same time and transferred to the same piece of PVDF membrane. The membrane was incubated with mixture of primary antibodies against p53 (pAb1801 and pAb240) and against TBP (3G3). After incubation with biotinylated secondary antibody and avidin-FITC, fluorescent signal was quantitated with FluorImager and ImageQuaNT software. 43 kD band of TBP can be easily separated from p53 bands and was used as internal standard for determination of total amount of protein. Ratio between signals from p53 and TBP bands was further divided with percentage of transfection efficiency of particular series of transfection. Result is given on Figure 3 and reflects ratio between signals of p53 and TBP bands per transfected cell. This result can be used only for comparison of protein amount on different lanes of the same blot and can not be interpreted as intracellular molar ratio between p53 and TBP, because the latter depends on concentration and affinity of different antibodies. As seen on Figure 3, the same amount of expression plasmid produced roughly the same amount of different p53 proteins and amount of each protein correlated with amount of expression plasmid used in transfections. Exception was ΔI, which had higher steady-state level at every concentration point. Diminished degradation can be reason for elevated level of ΔI protein, which has been

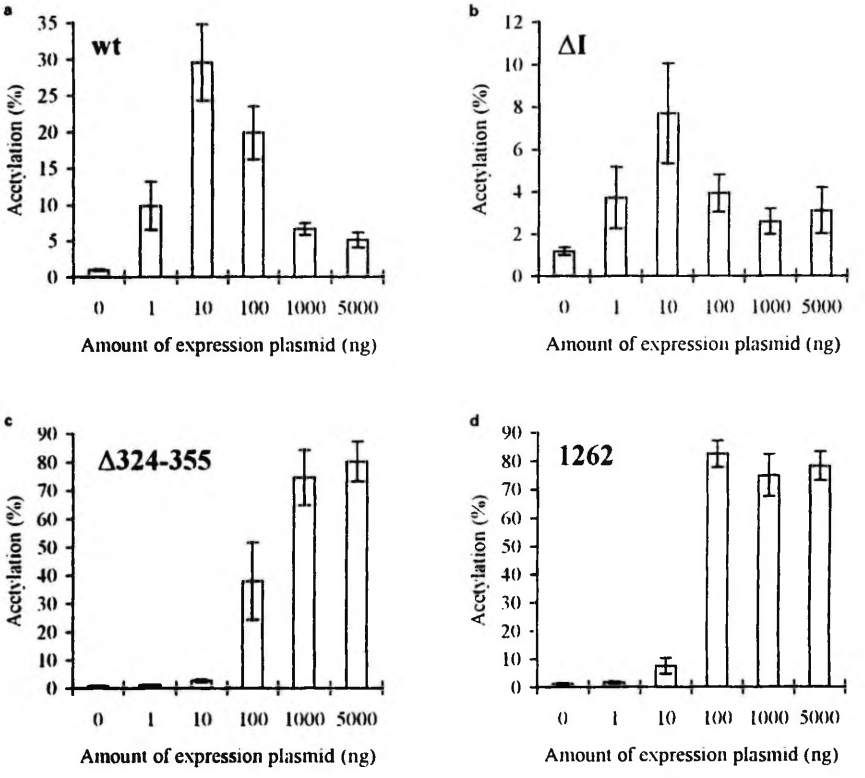

Figure 2 Activation of transcription by p53 proteins at different concentrations. Saos2 cells were cotransfected with 1 μg of CAT reporter plasmid and different amounts of p53 expression plasmids and were lysed 24 h after transfection. All lysates were normalized before CAT reaction according to total amount of protein (Bradford, 1976) and efficiency of transfection, thus every reaction contained the same amount of transfected cells. Three independent series of CAT assays with each p53 construct were quantitated with PhosphorImager and ImageQuaNT software. **(a)** wt p53; **(b)** ΔI; **(c)** Δ324–355; **(d)** 1262. The amount of p53 expression plasmid is indicated on abscissa of each diagram (in nanograms). The ordinate shows the percentage of chloramphenicol acetylation. Standard error is indicated by error bars. CAT reporter plasmid contains the *XhoI–XbaI* fragment from plasmid E2-100 CAT (Tarunina and Jenkins, 1993) in pBluescript KS$^-$. It has p53 consensus DNA-binding site CON (Funk *et al*., 1992) and modified adenovirus E2 promoter in front of chloramphenicol acetyltransferase gene. Efficiency of transfection was determined by parallel transfection with β-galactosidase expression plasmid pON260 (Spaete and Mocarski, 1985)

shown to be resistant to MDM2-directed degradation and is more stable when expressed in cells (Kubbutat and Vousden, 1997; Kubbutat *et al*., 1997). Quantitation of proteins also shows that weak activation of reporter gene by ΔI is not caused by lack of protein expression.

To test whether changes in specific DNA binding activity of p53 are responsible for loss of transactivation at high expression level, we tested the DNA-binding properties of wt p53 expressed at different concentrations. As seen on Figure 4, amount of specific DNA bound to wt p53 correlates with amount of p53 expression plasmid used in transfections indicating that low transactivating activity of p53 is not caused merely by low proportion of functional protein in the pool (compare CAT activity on Figure 2a and DNA-binding activity on Figure 4 lines 1 to 6). Retarded band seen on Figure 4 is specific to p53 because it can be titrated out with 25-fold molar excess of unlabelled CON site but not with nonspecific oligonucleotide (Figure 4, lines 6 to 8). It also can be supershifted with pAb1801 antibody reacting with N-terminus of p53 but not with pAb419 antibody directed against SV40 large T antigen (Figure 4, lines 4, 9, 10). pAb1801 does not produce any bands when cell lysate does not contain p53 (Figure 4, line 11).

Previous studies have shown that expression level can be a factor modulating the transactivating properties of p53. p53 accumulates efficiently after U.V. induction in mouse NIH3T3 and T22 cells. It has, however, been demonstrated that there is a discordance between the transcriptional activity of p53 and its protein level: expression of p53 target genes is induced only in cells, which contain moderate level of p53 protein and no gene expression is observed at high protein concentration (Lu *et al*., 1996). We have shown with reporter systems that the transactivation capability of wt p53 is strongly inhibited by high protein

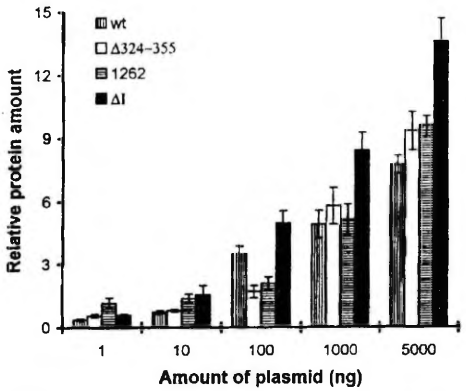

Figure 3 Quantitation of relative amount of p53 proteins. Amount of p53 protein was determined in every transfection. Fluorescent signal from p53 and TBP bands of Western blot were quantitated with FluorImager. Diagram shows ratio between p53 and TBP bands per transfected cell (ordinate) at different amounts of expression plasmid used in transfections (abscissa). Columns show the average value, error bars indicate standard error. Cells were lysed 24 h after transfection in SDS loading buffer (50 mM Tris-HCl pH 6.8; 100 mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol) and boiled for 10 min. Lysate was separated by SDS–PAGE in 10% gel. Proteins were transferred to BioBlot-PVDF membrane ('Costar'). Free surface of the filter was blocked for 10 min at RT with 1% non-fat milk powder in PBS+0.05% Tween 20. Membrane was incubated with monoclonal antibodies 3G3, pAb1801 and pAb240 in blocking solution for 2 h at RT. After washing with PBS+Tween 20, filter was incubated for 1 h at RT with biotinylated secondary antibody against mouse Ig. Finally, the membrane was incubated with streptavidin-FITC. Quantitation of data was performed with FlourImager and ImageQuaNT software ('Molecular Dynamics')

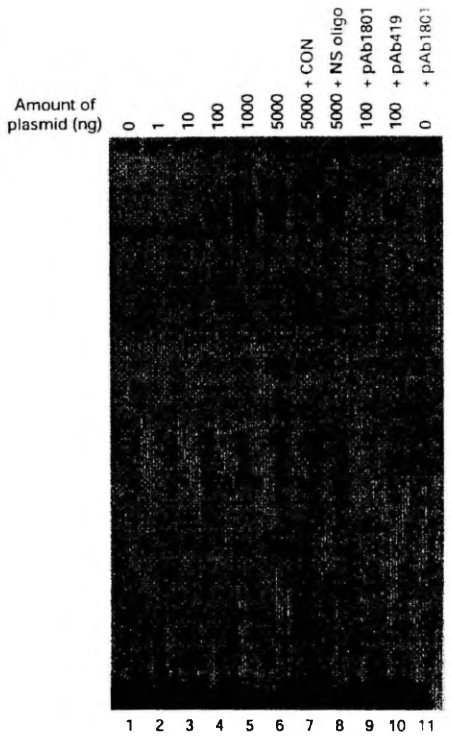

Figure 4 DNA binding activity of wt p53 at different concentrations. Lysates from cells transfected with different amounts of pCG-Hwt were incubated with 2 ng of radiolabeled CON site in buffer containing 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% NP40, 5% sucrose, 200 ng herring sperm DNA and 2 μl pAb421 supernatant. Amount of expression plasmid used for transfection is indicated on top of lanes. 50 ng of unlabeled CON site or nonspecific ds-oligonucleotide was added to reactions on lanes 7 or 8, respectively. Five μl of pAb1801 or pAb419 supernatant was added as indicated. Protein-DNA complexes were separated in 5% TBE polyacrylamide gel and exposed to X-ray film

concentration in the cells (Kristjuhan and Maimets, 1995). In this study we specified the structural requirements of this finding. We showed that oligomerization of p53 protein is needed for loss of transactivation at high p53 concentrations. The highest transactivating activity of oligomerization-competent wt and ΔI proteins was observed at relatively low concentrations of p53 protein. Increasing p53 concentration causes inhibition of transactivation when compared with lower protein amounts. This effect was not observed with monomeric p53 proteins. We also tested p53 mutant 517 (Tarunina and Jenkins, 1993), which forms preferably dimers but also higher oligomers in solution (Stürzbecher *et al.*, 1992). The level and dynamics of transactivation by 517 was the same as with wt p53 (data not shown). We conclude that oligomerization of p53 is important to suppress transactivation at high concentrations.

Mechanism of transactivation by p53 is most likely based on interactions with proteins of the basal transcription machinery. To date it has been shown that p53 interacts with TFIIH and TFIID complexes (Liu *et al.*, 1993; Xiao *et al.*, 1994), with TBP (Martin *et al.*, 1993; Seto *et al.*, 1992; Truant *et al.*, 1993), TBP associated proteins $TAF_{II}60$ and $TAF_{II}40$ from *Drosophila* cells (Thut *et al.*, 1995), and with human analogue of *Drosophila* $TAF_{II}40$, $TAF_{II}31$ (Lu and Levine, 1995). At overexpression conditions, activator proteins could titrate out some basal transcription factors, amount of which is limited in the cell. This phenomenon is known as squelching (Ptashne, 1988). At optimal concentrations, every activator molecule bound to the test promoter, is also bound to the target molecule(s) (for example TFIID complex). In excess of the activator, most of activator proteins bound to the test promoter are not bound to the target molecule(s) because of limited amount of the latter. Therefore, activation of the test promoter is inhibited as compared to the optimal concentration of activator. In the case of p53, *in vitro* experiments have shown that TFIID complex and TFIIB protein are the limiting factors, which can be titrated out by excess amounts of p53 (Liu and Berk, 1995). It is therefore possible that inhibition of transactivation by wt and ΔI proteins at high concentrations is caused by squelching of some basal transcription factors. On the other hand, high levels of monomeric mutants Δ324–355 and 1262 did not cause the effect of squelching.

One possible explanation here is that monomeric p53 can not interact with transcription machinery at all and therefore it can not squelch basal transcription factors. Analogously, oligomeric state of p53 is needed for interaction with MDM2 (Marston *et al.*, 1995). Although specific complex between DNA and monomeric p53 is poorly detectable in cell lysate, purified p53 monomers are capable to bind cooperatively to consensus DNA (Balagurumoorthy *et al.*, 1995; Wang *et al.*, 1995). p53 binding DNA consensus sequence contains two tandem decameric elements, each containing two pentameric inverted repeats. Therefore, the DNA itself can be a factor, which brings four p53 monomers together and p53 could gain the ability to interact with transcription machinery only after binding to the DNA consensus sequence. Increasing concentration of monomeric p53 in the cell could increase probability that more test promoters are bound to multiple p53 monomers, which in turn assures higher transcription efficiency from the promoter.

In this paper we have shown that the ability of p53 to activate transcription may be regulated by its state of oligomerization. In that sense, p53 is similar to another transcription factor, *Drosophila* protein Krüppel (Kr). Monomeric Kr can act as a transcriptional activator, whereas Kr oligomers formed at high concentrations cause repression. Interactions with different parts of transcriptional machinery are responsible for these effects. (Sauer *et al.*, 1995).

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III

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Tumour associated mutants of p53 can inhibit transcriptional activity of p53 without heterooligomerization

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Tumour suppressor protein p53 is the most frequent target of mutations occurring in different types of human cancers. Most of these are point mutations clustered in certain 'hot spots'. Because p53 is a tetramer in solution, it can form heterooligomers when both wild-type and mutant forms of p53 are expressed in the same cell. Inactivation of wt p53 by heterooligomerization has been proposed as a mechanism for dominant negative effect of mutant protein. In this paper we show that other mechanisms can also be involved in the inhibition of transcriptional activity of wt p53 by mutant proteins. In addition to suppressing the wt p53 activity, mutant proteins are also able to suppress the activity of p53 protein unable to oligomerize. Either N- or C-terminus of mutant p53 are needed for this activity. The suppression of transcriptional activity described is restricted to p53-dependent promoters and no effect is seen with the promoter not containing p53 binding site. Point mutants also inhibit the growth suppressing activity of monomeric p53. Our data allow to propose the existence of a cofactor specifically needed for p53-dependent transcription. Depletion of this cofactor could be an alternative mechanism of inactivation of wt p53 by its point mutants.

Keywords: p53; transcription; oligomerization

Introduction

Tumour suppressor protein p53 has been shown to be involved, either directly or indirectly, in many cellular activities. It controls transcriptional activation from promoters containing its binding site (Funk *et al.*, 1992; El-Deiry *et al.*, 1992) and the translation of its own mRNA (Mosner *et al.*, 1995). p53 also has direct effect on DNA replication in embryonic cells (Cox *et al.*, 1995). It is involved in the control of cell cycle (Levine *et al.*, 1991) and apoptosis (Lowe *et al.*, 1993).

Of all these activities, the regulation of transcription by p53 has been most extensively studied. p53 binds specifically to DNA similar or identical to the consensus 5'RRRC(A/T)(A/T)GYYYN$_{0-13}$RRRC(A/T)(A/T)GYYY3'(El-Deiry *et al.*, 1992). There are several genes containing this type of p53 binding site in their promoters and are therefore regulated by changes of levels of p53. These include p21$^{WAF1/CIP1}$ (El-Deiry *et al.*, 1993), mdm2 (Barak *et al.*, 1993), gadd45 (Kastan *et al.*, 1992), bax (Miyashita and Reed, 1995) and IGF BP-3 (Buckbinder *et al.*, 1995).

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Human p53 protein, which contains 393 amino acids, is able to form tetramers due to the protein-protein interactions. An area responsible for tetramerisation is located between amino acids 323 and 355 (Clore *et al.*, 1994; Wang *et al.*, 1994). It has been shown that oligomeric p53 is more active in transcription, but monomeric forms of p53 are also able to activate promoters containing p53 binding site (Tarunina and Jenkins, 1993; Wang *et al.*, 1993; Crook *et al.*, 1994; Sang *et al.*, 1994; Pellegata *et al.*, 1995). Although monomeric p53 does not form oligomers in solution, it still binds DNA co-operatively as a tetramer (Balagurumoorthy *et al.*, 1995).

In human tumours p53 protein has been often mutated. Because it is expressed from both alleles, the overall content of intracellular p53 can be either wild-type (wt), mixture of wt and mutant p53 or mutant p53 only (when both alleles are mutated or one allele is deleted). Although the tumour-associated mutations found cover the whole molecule, certain 'hot spots' for mutations have been described (Levine *et al.*, 1991). These point mutants have lost their DNA-binding activity. In the case of coexpression of mutant and wt p53 it has been shown that mutant p53 can drive wt p53 to mutant conformation (Milner and Medcalf, 1991) and the transactivation function of wt p53 is inhibited by mutant p53 (Kern *et al.*, 1992). It is generally believed that the mechanism behind this effect is that mutant p53 proteins form heterooligomers with wt proteins, which are less active in transcriptional activation than homooligomers of wt protein.

In this paper we show that, in addition of heterooligomerization, other mechanisms of repression of p53 function can also be involved in cells expressing both wild-type and mutant p53. We show that (1) the transcativation function of p53 protein unable to oligomerize is also suppressed by tumour-associated mutants of p53; (2) This effect is specific to p53-dependent promoters and requires the presence of either C- or N-terminus of the mutant protein; (3) Tumour-associated point mutants are able to inhibit the growth suppressing function of monomeric p53. Our data allow to propose the existence of p53-specific transcriptional coactivator(s) needed for p53 to activate the p53-specific promoters and mediate its biological effects.

Results

Tumour-associated point mutants of p53 can inhibit the transactivation function of monomeric p53

Mutant p53 has a dominant negative effect over the wt protein. This is thought to be mediated through the

heterooligomerization between wt and mutant p53. In that case, the monomeric p53 would be insensitive to mutant protein, because heterooligomers cannot form. To test this hypothesis, we carried out series of transfections (Figure 2). Every transfection contained 1.2 μg of pBS-CON-CAT as a reporter and 250 ng of Δ324–355 as a transactivator (except the first column, which represents transfection, containing only the reporter). Cells were lysed after 24 h and CAT assays were performed. Cotransfection with different point mutants clearly inhibited the activity of Δ324–355 (Figure 2a). To get more insight into the nature of this inhibition, we made several deletions based on the Trp248 mutant (Figure 1). When either N- or C-terminus are deleted, the mutant can still inhibit the transcriptional activity of the Δ324–355 (Figure 2B). When both of these termini are absent (40-361Trp), the mutant is not able to repress the Δ324–355 activity. Neither of the point mutants used had any transcriptional activation function by themselves (data not shown).

Repression phenomenon is not dependent on the nature of the p53 binding site

Reporter plasmid, used in the experiments described in Figures 2a and b, 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. To test our finding in more physiological context, we did the same kind of experiments using a plasmid pWWP-CAT, containing 2.4 kb fragment from human WAF1 promoter in front of the CAT gene (El-Deiry *et al.*, 1993). Figure 3 shows the results of three independent experiments. Every transfection contained 1.2 μg of pWWP-CAT as a reporter and 5 μg of pCG-Δ324–355 as a transactivator (except the first column, were only the reporter plasmid was used for transfection). Note that we used much higher amount of pCG-Δ324–355 than in previous experiment in order to get sufficiently high CAT activity. This was probably due to weaker binding of p53 to its binding site in WAF1 promoter as compared to the CON site. Different point mutants repress the transactivation function of the Δ324–355 with the same pattern as in the Figures 2a and b – only the 40-361Trp mutant was not able to inhibit the

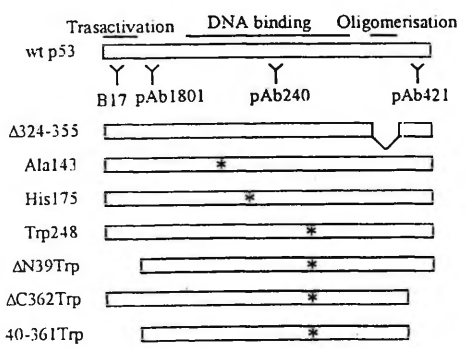

Figure 1 Schematic representation of deletion mutants of p53. Asterisks indicate point mutations. Functional domains and epitopes for antibodies are indicated

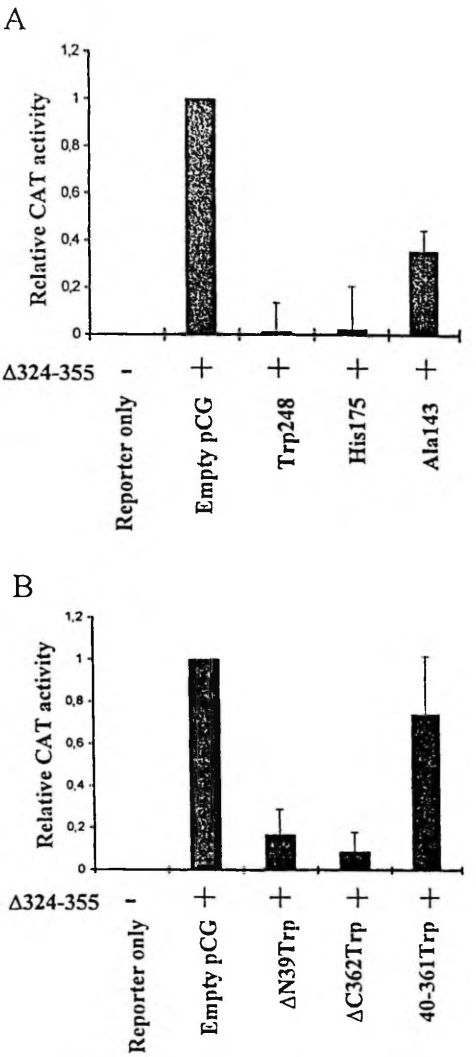

Figure 2 Mutant p53 proteins inhibit transactivation of monomeric p53. (a) Cells were cotransfected with Δ324–355 (250 ng) p53 expression vector and with expression plasmid carrying point mutant p53 cDNA or with empty pCG plasmid (5 μg). 1.2 μg of pBS-CON-CAT reporter plasmid was included in every transfection. Columns indicate average CAT activity from three independent experiments. CAT activity from cotransfection with empty pCG vector is taken as 1. (b) Cells were cotransfected with Δ324–355 (250 ng) p53 expression vector and with the expression plasmid carrying deletions of Trp248 mutant (5 μg). 1.2 μg of CAT reporter plasmid was included in every transfection. Columns indicate average CAT activity from three independent experiments. CAT activity from cotransfection with empty pCG vector is taken as 1

transcription. We also used a reporter with p53 binding site from mdm2 promoter and this also behaved similarly (data not shown). We conclude that inhibition of transactivation function of monomeric p53 by point mutant occurs from different p53 binding sites and does not depend on promoter structure.

p53-independent transcription is not affected by point mutants

Wt p53 can, at least when overexpressed, inhibit many different promoters. Though there are no hints in the literature that tumour-associated point mutants can do the same, we tested their ability to inhibit the CMV promoter. This is a strong eukaryotic promoter, containing no p53 binding sites. A plasmid, containing β-galactosidase gene under the control of CMV promoter was included in every transfection. After the lysis of the cells half of lysate was used to determine the β-galactosidase activity. As the Figure 4 shows, tumour associated point mutants do not affect the β-galactosidase activity. Therefore, the inhibitory effect of the point mutants is restricted to the promoters containing p53 binding site.

Coexpression of point mutants with Δ324–355 does not alter the quantity or localisation of the latter

One possibility to explain why the point mutants inhibit the activity of the Δ324–355, is that their own expression may saturate some stage of the gene expression and therefore the expression of the Δ324–355 decreases. To control this, we analysed by western blotting the lysates from cells contransfected with Δ324–355 and different point mutants (Figure 5). No remarkable differences in the amount of Δ324–355 can be observed between different transfections. Therefore, the differences in the transcriptional inhibition function between ΔN39Trp and 40-361Trp or between ΔC362Trp and 40-361Trp can not be explained by different expression levels of Δ324–355. Note that ΔC362Trp and Δ324–355 comigrate as a one band.

In order to function as transcriptional activator p53 must be localised in cell nucleus. It could be possible that expression of the point mutants will abolish the nuclear localisation of the Δ324–355. To control this we cotransfected the cells cotransfections with pCG-Δ324–355 and with plasmids encoding deletions of point mutants. Immunoflorescence analysis with different antibodies detected only the Δ324–355

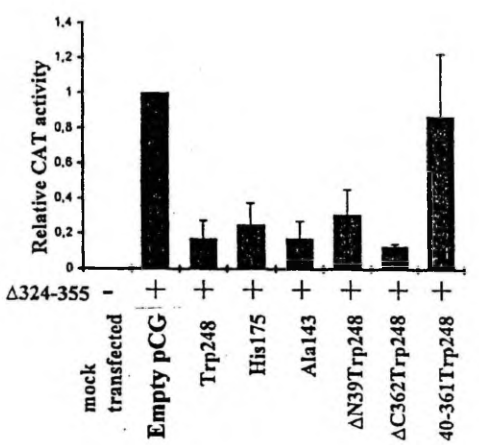

Figure 3 Inhibition of transactivation occurs also from natural promoters. Cells were cotransfected with Δ324–355 (5 μg) p53 expression vector and with expression plasmid carrying point mutant p53 cDNA, deletions of Trp248 mutant or with empty pCG plasmid (5 μg). 1.2 μg of pWWP-CAT reporter plasmid was included in every transfection. Columns indicate average CAT activity from three independent experiments. CAT activity from cotransfection with empty pCG vector is taken as 1

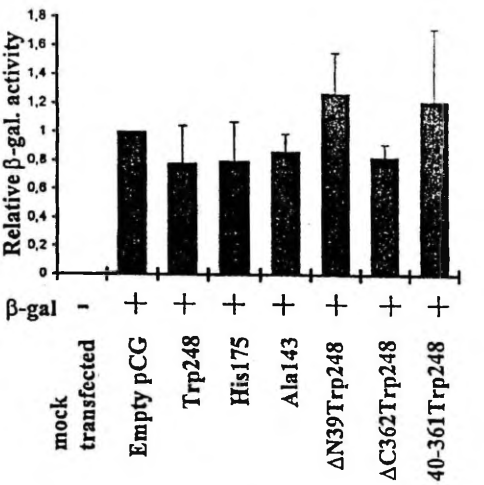

Figure 4 Point mutant p53 proteins do not inhibit p53-independent transcription. All transfections, were Δ324–355 was used as activator, contained 0.5 μg of β-galactosidase reporter plasmid with CMV promoter. Columns indicate average β-galactosidase activity from three independent experiments. Activity from cotransfection with empty pCG vector is taken as 1

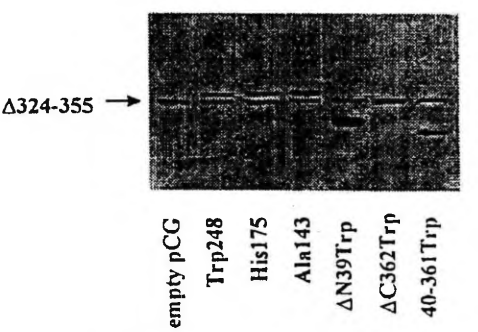

Figure 5 Coexpression with different deletion mutants does not alter the amount of Δ324–355. Cells were cotransfected with 5 μg pCG-Δ324–355 and with 5 μg of the constructs indicated. After 24 h cells were lysed and the Western blot was carried out. p53 was detected with monoclonal antibody pAb240

protein in cells expressing two forms of p53. As shown in Figure 6, the Δ324–355 is nuclear in all cases examined and the inability to localise in nucleus is not the reason for transcriptional inhibition.

Δ324–355 does not form heterooligomers with p53 point mutants

To show that protein Δ324–355 is not able to give oligomers in the expression conditions used here we carried out crosslinking experiments. Cells cotransfected with Δ324–355 and mutants ΔN39Trp, ΔC362Trp or 40-361Trp were lysed, crosslinked with glutaraldehyde, devided between two equally loaded gels, electrophoresed and blotted to nitrocellulose. Using different primary antibodies we were able to show that even in the case of coexpression with point mutant the Δ324–355 remains monomeric in solution (Figure 7). From this we conclude that transcriptional inhibition by ΔN39Trp and ΔC362Trp is not due to heterooligomerization with Δ324–355.

Point mutants can inhibit the growth suppression function of monomeric p53

In order to clarify the relevance of our results in more biological assay, we tested the influence of point mutants to the growth suppression activity of Δ324–355. It is reported that monomeric p53 expresses clear growth suppressing activity, although less than wt protein does (Shaulian *et al.*, 1993; Thomas *et al.*, 1996). We performed growth suppression assay and results of that are described in Figure 8. Wt p53 inhibited colony formation almost entirely and Δ324–355 reduced the number of colonies approximately 50% as compared to the transfections with the resistance marker (pBabe Puro) only. When Trp248, ΔN39Trp or ΔC362Trp were added to the transfection mixture, they inhibited the growth suppressing activity of the monomer. The same mutants did not affect notably the colony formation activity, when expressed alone with pBabe Puro (data not shown). Because the 40-361Trp has itself the ability to reduce the colony number (data not shown), its effect to the activity of the monomer could not be examined.

Discussion

Transcriptional activation is the best studied biochemical function of p53. p53 mediates signals for cell cycle arrest and apoptosis by activating transcription from many different promoters (Kastan *et al.*, 1992; El-Deiry *et al.*, 1993; Barak *et al.*, 1993; Miyashita and Reed, 1995; Buckbinder *et al.*, 1995). Tumour hot-spot mutations occur at residues essential for DNA-binding and therefore inactivate the transcriptional activation function of p53 (Kern *et al.*, 1992). Because most of wt p53 exists in tetrameric form, heterooligomers between wt and mutant p53 are present in tumours. Inactivation of wt p53 by heterooligomerization with mutant protein is thought to be the mechanism of inhibition of wt p53 activities by mutant p53, transcriptional activation included. However, we present here experimental evidence showing that heterooligomerization is not the only way how mutant p53 can inhibit wt p53

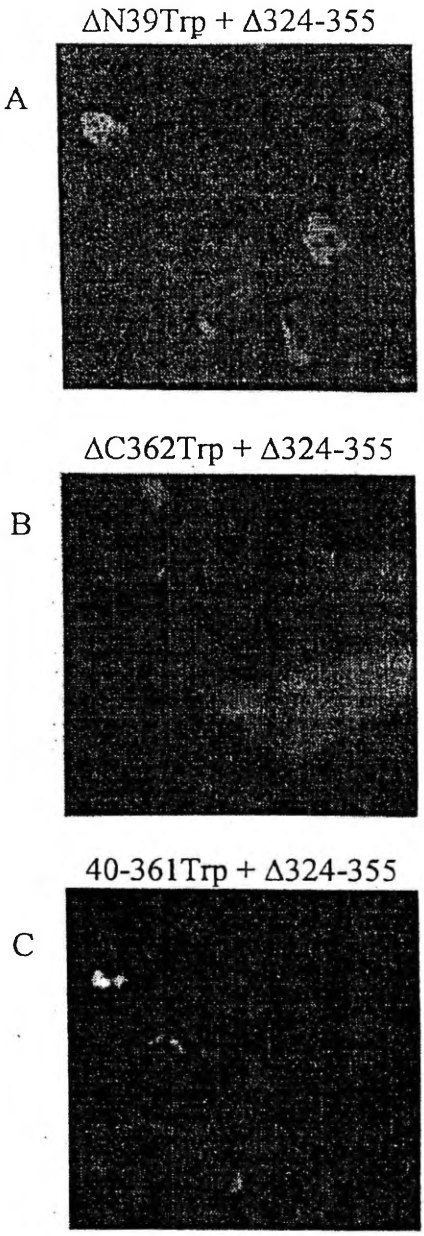

Figure 6 Δ324–355 is nuclear when coexpressed with point mutants. Cells were transfected with the 5 μg pCG-ΔN39Trp (a), pCG-ΔC362Trp (b) or pCG-40-361 Trp (c). 5 μg pCG-Δ324–355 was included in all transfections. In a and c cells were stained with antibody B17, which reacts with p53 amino acids 16–25 (epitope deleted in ΔN39Trp and in 40-361Trp). In b cells were stained with antibody pAb421, which reacts with p53 amino acids 372–382 (epitope deleted in ΔC362Trp). With these antibodies only the Δ324–355 is detected. Transfection efficiency was approximately 25%

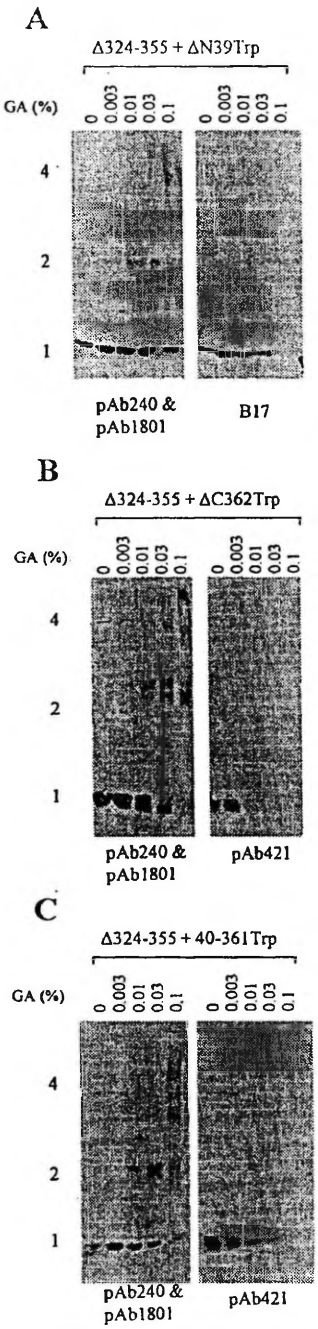

Figure 7 Δ324–355 does not form heterooligomers with point mutants. Cells were transfected with 2 μg pCG-ΔN39Trp (a), pCG-ΔC362Trp (b) or pCG-40-361Trp (c). 2 μg pCG-Δ324–355 was included in all transfections. Cells were lysed and proteins crosslinked with glutaraldehyde as described in Materials and methods. Every sample was divided in two, electrophoresed and blotted on nitrocellulose. Antibodies pAb240 and pAb1801 were used to detect all forms of p53. In duplicate lanes, the antibody recognising only the Δ324–355 was used (a B17; b and c pAb421). Bands corresponding to mono-, di- and tetramers are indicated on left side (1, 2 and 4, respectively)

transactivation function. Mutant forms of p53 can also inhibit the transactivation function of monomeric p53 (Δ324–355; Figure 2, see also Subler *et al.*, 1994). When both N- and C-termini are deleted from mutant p53, it is not able to inhibit Δ324–355 (Figure 3). The proof that point mutants can also affect the biological activity of the monomeric p53 comes from growth suppression assay (Figure 8). Expressing point mutants together with Δ324–355 clearly inhibited the growth suppression function of the latter. Taken together, our data suggest that in addition to heterooligomerization also other mechanisms can stay behind the inhibition of wt p53 by its mutant forms.

As a transcriptional activator p53 needs to bind several basic transcription factors. Indeed, interactions between p53 and components of TFIID and TFIIH complexes have been described (Liu *et al.*, 1993; Lu and Levine, 1995; Martin *et al.*, 1993; Seto *et al.*, 1992; Thut *et al.*, 1995; Truant *et al.*, 1993; Xiao *et al.*, 1994). It could be possible that mutant p53 binds some of these basic factors and depletes the cells from them. Our results show that this is not the case: overexpression of mutant p53 does not alter the expression from CMV promoter (Figure 4), activity of which is also dependent on general transcription factors. Overexpression of mutant p53 has influence only to p53-dependent promoter and this is not caused by alterations in Δ324–355 expression or localisation

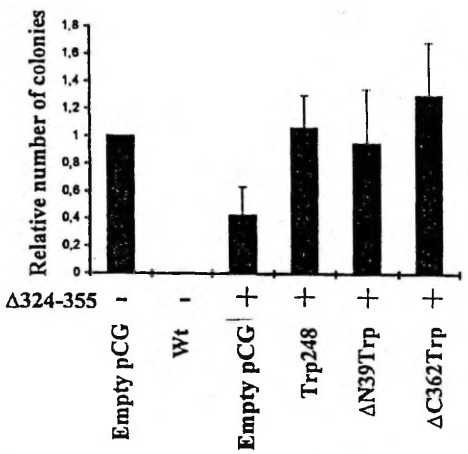

Figure 8 Point mutants can inhibit the growth suppression function of the Δ324-355. Cells were cotransfected with pBabe Puro (2 μg; contains the puromycin resistance gene between MuLV LTRs), pCG-Δ324–355 (5 μg; except column 1 and 2) and with 5 μg of plasmids encoding proteins indicated on graph. Twenty-four hours after transfection cells were replated (1.5×10^6 cells per plate); selection period for puromycin resistant colonies was 2 weeks. Columns indicate the relative amount of colonies (pBabe Puro only = 1; average of three independent experiments)

(Figures 5 and 6). From these data we propose that mutant p53 interacts with factor(s) specifically needed for p53-dependent transactivation but not for transcription in general.

Most tumour-associated mutations in p53 are clustered in the central part of the molecule and they inactivate the DNA binding function of the p53. Some of these mutations affect the overall conformation of central part of the molecule (Gannon *et al.*, 1990), whereas conformations of the N- and C-termini are most probably not changed. Therefore, the modifying enzymes and cofactors targeted to the termini of wt molecule are also able to bind to mutant p53. If the mutant form of p53 is expressed at high level, it may deplete cells from cofactors, necessary for p53 to activate transcription. The transactivating function of p53 can be allosterically modified by different factors. It has been shown that DNA-binding of wt p53 can be enhanced by specific peptides and antibodies against the C-terminus (Hupp *et al.*, 1992, 1995). The c-Abl and WT-1 are cellular proteins binding wt p53 and activating its transactivation function (Goga *et al.*, 1995; Maheswaran *et al.*, 1993, 1995; Yuan *et al.*, 1996). Recently, new activators of p53 were identified, including ref-1 (Jayaraman *et al.*, 1997), HMG-1 (Jayaraman *et al.*, 1998), p33^{ING1} (Garkavtsev *et al.*, 1998) and BRCA1 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). It is not known, whether they can interact with point mutants of p53 or with monomeric p53. Therefore we have no clues to speculate, could one of these activators be depleted from cells by mutant p53 in our experiments. Necessity of coactivator for p53-dependent transactivation is shown only for p33^{ING1}. Whether the others are also necessary for p53-dependent transactivation or have only additive effects needs to be clarified in future.

Two explanations can be proposed to the fact that 40-361 Trp cannot inhibit Δ324–355 mediated transactivation. First, the deletions of both ends in 40-361 Trp may affect the overall conformation of the molecule making it unable to bind the specific cofactor. The other explanation comes from hypothesis according to which N- and C-termini are close to each other in 3-dimensional structure. They could generate a binding site for this proposed cofactor and only the deletions of both of them abolish the binding of the cofactor.

Alternatively, wt p53 may need a specific modification for its activity rather than binding to a specific cofactor. Indeed, many kinases have been shown to phosphorylate p53. Casein kinase II and protein kinase C phosphorylate the C-terminus of p53 and probably unmask the DNA-binding function of p53 (Baudier *et al.*, 1992; Takenaka *et al.*, 1995; Meek *et al.*, 1990). S and G_2/M cdks can also activate p53 and even influence its binding site specificity (Wang and Prives, 1995). In addition to C-terminal phosphorylation sites, many serine residues which can be phosphorylated are clustered in N-terminus (Milne *et al.*, 1992; Knippchild *et al.*, 1996; Lees-Miller *et al.*, 1992). Replacing two or more of these serine residues with alanines diminishes p53 transactivating abilities (Mayr *et al.*, 1995). It is also known that wt p53 purified from bacteria does not bind DNA, although p53 produced in insect or mammalian cells does (Hupp *et al.*, 1992; Wolkowicz *et al.*, 1995). Possible explanation for this is that wt p53 from bacteria does not have necessary modifications. If p53 needs to be phosphorylated in order to activate transcription, the inhibitory effect of point mutants to Δ324–355 mediated transcription may relay on the saturation of the modifying enzymes. Because point mutants are expressed in high amounts they could titrate the Δ324–355 out. Following this line two or more modifications are needed for transactivation by p53 – at least one for N- and the other for C-terminus. If even one of these possible modifying enzymes is depleted from wt p53, the p53 cannot activate transcription. Only in the case of 40-361 Trp the Δ324-355 retains its activity, because this deletion of point mutant is not able to bind any modifying enzyme.

Materials and methods

Plasmid constructs and antibodies

CAT reporter plasmid pBS-CON-CAT contains the *XhoI*–*XbaI* fragment from plasmid E2-100 CAT (Tarunina and Jenkins, 1993) in pBluescript KS$^-$. It has p53 consensus DNA-binding site CON (Funk *et al.*, 1992) and modified adenovirus E2 promoter in front of CAT gene. pWWP-CAT contains 2.4 kb region from human WAF1 promoter (El-Deiry *et al.*, 1993) in front of the CAT gene and was a generous gift from Dr. Bert Vogelstein. Plasmid pON 260 (Spaete and Mocarski, 1985) was used to express β-galactosidase from CMV promoter. pBabe Puro contains the puromycin resistance gene under the control of SV40 promoter between the Murine Leukemia Virus Long Terminal Repeats (Morgenstern and Land, 1990). All p53 cDNA-s were cloned between *XbaI* and *BglII* sites of eukaryotic expression vector pCG (Tanaka and Herr, 1990). pCG-Hwt, pCG-His175, pCG-Ala143 and pCG-Trp248 contain human wt, 175 Arg→His, 143 Val→Ala and 248 Arg→Trp point mutation p53 cDNAs, respectively. For creating pCG-Δ324–355, two regions of wt p53 cDNA were amplified: one between codons 1 and 323 and another between codons 356 and 393. Resulting cDNA-s were linked in frame to each other from *PstI* site designed into PCR primers. Half of *PstI* site (CAG) encodes an extra amino acid (Glu) between these two regions. This cDNA was inserted between *XbaI* and *BglII* sites of pCG vector and encodes p53 protein with amino acids 324–355 deleted. ΔN39Trp encodes p53 protein with deletion of the first 39 amino acids and 248 Arg→Trp mutation. ΔC362Trp encodes truncated protein with stop codon at position 362 and 248 Arg→Trp mutation. 40-361Trp encodes mutant p53 starting from amino acid 40, containing a stop codon at the position 362 and amino acid substitution at position 248. Schematic presentation of p53 proteins is shown on Figure 1. Empty pCG does not contain any insert – the poly (A) signal follows directly the CMV promoter.

p53 was detected with monoclonal antibodies pAb421 (Harlow *et al.*, 1981, epitope between amino acids 372–381), pAb1801 (Banks *et al.*, 1986, epitope between amino acids 46–55), pAb240 (Gannon *et al.*, 1990, epitope between amino acids 210–214) and B17 (Legros *et al.*, 1994, epitope between amino acids 16–25). The last was generous gift from Dr. Thierry Soussi.

Cell culture and transfection

Saos2 cells were cultured in Iscove's modified Eagle's medium (IMEM) supplemented with 10% foetal calf serum. Cells were maintained at 37°C in humidified 5% CO_2 atmosphere. Cells were transfected by electroporation

method as follows: 5–10 million cells were collected from 70% confluent cell dishes and suspended in IMEM containing 5 mM Na-BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid). 50 μg sonicated salmon sperm DNA, expression and reporter plasmids were added to 250 μl of cell suspension and the cells were transfected using 'Invitogene' electroporator (settings 1000 μF and 210 V). After electroporation, cells were resuspended in IMEM and transferred to growth conditions on 6 or 10 cm diameter cell culture dishes.

CAT and β-galactosidase assay

1.2 μg of pBS-CON-CAT or pWWP-CAT and 0.5 μg of pON 260 reporter plasmids were used per transfection. Cells were collected 24 h after transfection and lysed by three cycles of rapid freezing and thawing. All lysates were normalised before CAT and β-galactosidase reaction according to total amount of protein (Bradford, 1976). Enzyme reactions were performed at standard conditions (Sambrook *et al.*, 1989). For CAT assay thin layer chromatography plates were exposed to PhosphorImager and data were quantitated with ImageQuaNT software.

Western blotting and oligomerisation assay

For Western blotting cells were lysed 24 h after transfection in 30 μl IP buffer (500 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% NP-40, 0.02% NaN_3, aprotinin mTIU/ml, 0.1 mM PMSF) for 30 min in ice. Cell debris were removed by centrifugation at 12 000 r.p.m. for 5 min, 30 μl SDS loading buffer (50 mM Tris-HCl pH 6.8; 100 mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol) was added to lysate and it was boiled for 10 min. Lysate was separated by SDS–PAGE in 12% gel. Proteins were transferred to nitrocellulose filter. Free surface of the filter was blocked for 10 min at room temperature (RT) with 1% non-fat milk powder in PBS + 0.05% Tween 20. Filter was incubated with monoclonal antibody pAb240 in blocking solution for 2 h at RT. After washing with PBS + Tween 20 filter was incubated for 2 h at RT with goat antibody against mouse Ig conjugated with alkaline phosphatase.

For oligomerization assay cells were transfected with 2 μg of expression plasmid and lysed 24 h later in 150 μl IP buffer on ice for 30 min. Each lysate was divided between five tubes and glutaraldehyde was added to final concentration of 0; 0.003; 0.01; 0.03 or 0.1%. After incubation for 15 min on ice, equal volumes of 2 × SDS loading buffer were added. Samples were boiled for 10 min, separated in 6% SDS-polyacrylamide gel and transferred to nitrocellulose filter. p53 was detected with antibodies pAb1801, pAb421, pAb240 and rabbit anti-mouse Ig secondary antibody conjugated with alkaline phosphatase ('LabAs', Estonia).

Immunofluoresence

Cells, growing on cover slips, were fixed at −20°C in acetone/methanol (1:1). Primary antibodies were diluted in 1% BSA in PBS and incubated at RT for 1 h. After washing three times with PBS cover slips were incubated with goat anti-mouse antibody conjugated with FITC (LabAs). Cells were visualised and photographed using Olympus AH-2 microscope.

Cell growth suppression assay

Saos-2 cells were transfected with pBabe Puro, pCG-Δ324–355 and with indicated plasmids (Figure 8). Twenty-four hours after transfection the cells were replated with equal densities (1.5×10^5 cells per 100 mm plate). Forty-eight hours after transfection the fresh medium containing puromycin (1 μg/ml) was added. Cells were grown in the presence of puromycin for 1 week and then 1 week without the selection. After that cells were fixed in 0.05 glutaraldehyde in PBS, stained with Coomassie R-250, and the colonies were counted.

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