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TRANSACTIVATION AND DNA BINDING ACTIVITY OF MUTANT P53

by

AGUSTIN CHICAS

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2001

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ABSTRACT

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by

AGUSTIN CHICAS

Advisor: Professor Jill Bargonetti

p53 is the most commonly mutated tumor suppressor gene in human tumors. Mutants of p53 found in tumors have the peculiarity of not only losing the tumor suppressor activity but of also acquiring new growth promoting functions. It has been postulated that the growth promoting activity of mutants of p53 is due to the ability of these mutants to activate transcription of many growth promoting genes (Roemer, 1999). The mechanism of transactivation by the tumor derived mutants of p53, however, remains unclear. Unlike wild-type p53 which is a gene specific transactivator (Oren, 1999), mutants of p53 are generally defective in sequence specific DNA binding (Zambetti, 1993). Although some p53 mutants can bind to DNA albeit different than the wild type protein (Di Cuomo, 1998; Kern, 1991; Park, 1994; Niewolik, 1995), none of the mutants have been shown to bind to any of the genes that are transactivated by mutant p53.

We have used the HIV-LTR to study the mechanism of mutant p53 transactivation as this is one of the promoters that is transactivated by mutants of p53 (Subler, 1994b). Deletion analysis of the HIV-LTR have revealed that the three Sp1 binding sites of the HIV-LTR are important for mutant p53 transactivation (Subler, 1994). We have previously shown that purified mutant p53 does not bind to these three Sp1 binding sites (Bargonetti, 1997). Since Sp1 has been shown to associate with other transcription factor to activate transcription (Kardassis, 1999), we proposed and tested a model whereby mutants of p53 activate transcription by associating with DNA bound

Sp1 (Fig. 1.1). Using DNA affinity chromatography, we showed that mutant p53 His 273 in the MDA 468 cells formed a complex with Sp1 on HIV-LTR DNA. This complex, which is not detected by co-immunoprecipitation, is also formed on the p53 super consensus sequence (SCS). We showed that this endogenously expressed mutant p53 his 273, like transfected mutant p53, can activate HIV-LTR driven transcription (Chapter. 3). These data are consistent with our model. Together with previous results, these data suggest that the gain of function activity of mutant p53 may come from the ability of mutant p53 to associate with DNA bound Sp1. This association could lead to an increase in the expression of genes containing Sp1 binding sites. Interestingly, Sp1 binding sites are commonly found in housekeeping and growth promoting genes (Naar, 1998).

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A goal can be accomplished
It can never be done alone
Here I recognized
Those who cared

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DEDICATION

This thesis is rightly dedicated to my brother Felipe Antonio. I would have never accomplished this goal if it were not for him. I will never forget how he kindly decided to send me to school when I arrived in the United States in 1985. Unlike other immigrants who bring their relatives and put them to work, he sent me to school. This was the most pivotal moment in my life. Gracias hermano, tu has sido como un padre para mi.

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Chapter 1

Introduction

Originally characterized as an oncoprotein, today p53 is known for being the product of a potent tumor suppressor gene. This is evident from the fact that more than 50% of human tumors have inactivating mutations of p53 (Hollstein, 1991) and the observation that $p53^{-/-}$ mice develop tumors early in life (Donehower, 1992). The role of p53 as tumor suppressor is to maintain genomic integrity by sensing cellular stress and triggering a stress response that prevents the replication of damaged DNA. p53 prevents the replication of damaged DNA by causing growth arrest to give the repair machinery time to repair the damaged DNA or by killing those cells whose DNA has been damaged beyond repair (Agarwal, 1998). p53 executes these diverse functions, partly, by regulating transcription of a number of target genes (El-deiry, 1998).

The best characterized function of p53 is the gene-specific transactivation activity and different regions or domains of p53 have been defined in terms of the role they play in transactivation (Review by Prives, 1994). The activation domain, which is located at the amino terminus (Field, 1990), makes contact with members of the transcription factor IID complex such as the Drosophila $TAF_{II}40$ and $TAF_{II}60$ [Thut, 1995] and the human TAF31 (Lu and Levine, 1995) and other members of the general transcription machinery such as TBP and the p62 subunit of TFIIH (Review by Ko, 1996). This association of p53 with the TFIID complex is crucial for its transactivation activity as demonstrated by the fact that the mutant p53 Gly22-Ser23, which is unable to interact with this complex, can't activate transcription of wt p53 responsive genes (Lin, 1994). The sequence-specific DNA binding domain, which is located in the central region (Bargonetti, 1993; Paveletich, 1993), makes contact with DNA through amino acid residues R273, R283,

R248, R280 among others (Cho, 1994). p53 binds as a tetramer to two copies of the consensus sequence **5'-PuPuPuC(A/T)(T/A)GPyPyPy-3** comprising 4 inverted quarter sites in which one p53 monomer binds to one of these quarter sites (Cho, 1994). Variations of this sequence are found in a number of genes whose transcription is activated by p53 (El –deiry, 1998). The carboxyl terminus of p53 contains the non-specific DNA/RNA binding domains (Wang, 1993; Balkalkin, 1994); a domain that may be involved in recognition of damaged DNA (Reed, 1995; Lee, 1995). A domain that allows p53 to form oligomers is also located at the carboxyl terminus (Jeffrey, 1995). Finally, a domain that regulates the activity of the sequence-specific DNA binding domain is also found at the carboxyl terminus (Bayle, 1995). Modification of the carboxyl terminus such as deletion, phosphorylation or acetylation, and binding by carboxyl terminal-derived peptides, carboxyl terminal antibodies or the redox/repair protein (Ref-1) have been shown to activate the latent sequence–specific DNA binding activity of p53 (Review by Oren, 1999).

A less well characterized activity of p53 that may contribute to its role as a tumor suppressor is its transcriptional repression activity (Review by Ko, 1996). A number of viral and cellular promoters lacking p53 binding sites have been shown to be repressed by wild type p53 (Deb, 1992; Ginsberg, 1991; Subler, 1992; Subler, 1994b; Murphy, 1996; Kley, 1992; Pesch, 1996). Despite the wide range of promoters that are repressed by over-expressed p53, there appears to be some selectivity as TATA-less promoters were reported not to be responsive to repression by p53 (Mack, 1993). A repression domain has been identified in a region in the carboxyl terminus of p53 that associates with TBP

(Subler, 1994a; Horikoshi, 1995; Shaulian, 1995). It has also been shown that some of the domains required for activation of transcription are also required for repression of transcription (Subler, 1994a; Ragimov, 1993). Like transcriptional activation, repression of transcription requires a functional activation domain (Subler, 1994a) and a functional sequence-specific DNA binding domain (Ragimov, 1993). Unlike transcriptional activation which is enhanced by carboxyl terminal deletions, deletion of 75 amino acids at the carboxyl terminus of p53 (393-327) inhibits repression of transcription (Subler, 1994a). Other modifications of the carboxyl terminus such as phosphorylation are important for transcriptional repression for it has been shown that blocking the phosphorylation of Ser386 inhibits repression of transcription (Hall, 1996).

Regulation of stability and DNA binding

p53 levels and activity are tightly regulated in cells. In fact under normal physiological conditions, the p53 protein is not detected by conventional methods and appears to be in a latent, inactive form (Levine, 1997). This low protein level is due to a rapid protein turnover which is mediated by two proteins: the Mdm2 oncoprotein and the Jun N-terminal Kinase protein (JNK). While mdm2 binds to p53 in an region overlapping the activation domain (Oliner, 1993), JNK binds to the central region of p53 (Adler, 1997). Both of these proteins trigger the ubiquitination of p53 and its subsequent transport to the cytoplasm for degradation in the proteosome (Haupt, 1997, Kubbutat, 1997, Fuchs, 1998). The two proteins seem to share the duty by promoting degradation in different stages of the cell cycle as JKN-p53 complexes are detected in G0/G1,

meanwhile mdm2-p53 complexes are found in S/G2M (Fuchs, 1998). While mdm2 promotes the degradation of p53 by directly ubiquitinalating p53 (Fang, 2000, Honda, 1997), the mechanism by which JNK promotes p53 degradation is not known. Other factors can also contribute to the rapid turnover of p53. For example, the ubiquitous cytoplasmic proteases, calpains, have been shown to promote p53 degradation (Pariat, 1999). Yet, the most important player appears to be mdm2 as best illustrated in mdm2 knock out mice. Mdm2 knock out mice die early in embryogenesis. This early death appears to be caused by deregulated p53, for mice inactivated for both p53 and mdm2 are viable (Montes, 1995).

p53 can be released from this tight regulation that prevents its detection by a variety of signals including DNA damage, oncogenes, hypoxia, redox stress, and altered ribonucleotide pool (Review by Giaccia, 1997). These stimuli lead to the accumulation of p53 by preventing its degradation by mdm2 and to the activation of p53 as a gene specific transactivator. The mechanisms that regulate accumulation of p53 appear to be different from the mechanisms that regulate activation. Transactivation by p53, for instance, has been observed in the absence of any detectable increase in p53 protein level (Hupp, 1995). Similarly, accumulation of wt p53 can be achieved in the absence of any detectable transactivation activity (Chernov, 1998).

The biochemical pathways that lead to accumulation and activation of p53 differ for each type of stress. In the case of DNA damage, the pathways leading to accumulation and activation of p53 are different for different types of lesions. Ionizing radiation (IR),

which causes double strand breaks, stabilizes p53 by inducing phosphorylation at Ser-15 by the kinase ATM (Canman, 1998). This modification decreases the affinity of mdm2 binding and therefore blocks mdm2-mediated degradation (Shieh, 1997) leading to the accumulation of p53. IR, simultaneously activates the sequence-specific DNA binding activity of p53 by inducing modifications at the carboxyl terminus. These modifications include an ATM-dependent dephosphorylation of Ser 376 (Waterman, 1998) followed by the acetylation of Lys 382 (Sakaguchi, 1998).

Like IR, UV-radiation stabilizes p53 by inducing phosphorylation of Ser-15 but unlike IR, UV induced phosphorylation of Ser-15 is independent of the ATM kinase (Siliciano, 1997). Phosphorylation of ser15 in response to UV may require the ATM related kinase ATR or/and the extracellular signal-regulated protein kinase (ERKs). Both of these kinases are activated by UV and have been shown to phosphorylate p53 on ser15 (Tibbetts, 1999; She, 2000). Similar to IR, UV activates the sequence specific DNA binding activity of p53 by inducing modifications at p53's carboxyl terminus; although the modifications are somewhat different than those induced by IR. One of the modifications is the phosphorylation of Serine 392 (Kapoor, 1998, Lu, 1998); a site that has been shown to be phosphorylated by Casein Kinase II in vitro but it is not clear whether CKII phosphorylates this site in vivo (Hall, 1998). Another UV-induced modification of p53 is the acetylation of Lys382 (Sakaguchi, 1998).

The fact that both UV and IR induce acetylation of p53 at lys382 indicates that this is an important modification. *In vitro*, this site has been shown to be acetylated by the acetylase CBP/p300 (Gu, 1997).

The role of ser15 phosphorylation on the stabilization of p53 has been questioned recently. It has been shown that p53 ser15 mutants substituted with either Ala or Asp are stabilized following both IR and UV treatment indicating that ser15 phosphorylation is not required or sufficient to cause stabilization of p53. Instead, the same study shows that Ser20 mutants substituted with either Ala or Asp are not stabilized after DNA damage indicating that the phosphorylation of ser20 is required for stabilization of p53 (Chehab, 1999). Consistent with this report, a separate study showed that ser15 phosphorylated peptides but not ser20 phosphorylated peptides associated with mdm2 (Chehab, 1999). These data point to ser20 as the key residue for stabilization of p53. Ser20 has been shown to be phosphorylated by Chk2, a downstream target of the ATM kinase (Hirao, 2000). Consistent with a role of ser20 phosphorylation on the stabilization of p53, IR does not cause accumulation of p53 in Chk2 deficient thymocytes (Hirao, 2000). ATM therefore can induce phosphorylation of p53 by either directly phosphorylating ser15 or by activating Chk2 which in turn phosphorylates p53 at ser20. A recent report showed that the phosphorylation of ser15 is important for the transactivation activity of p53 but not its stabilization (Dumaz, 1999). Consistent with previous reports that UV-induced stabilization of p53 is independent of the ATM kinase pathway (Siliciano, 1997). p53 is stabilized in the Chk2 deficient mice in response to UV (Hirao, 2000).

Oncogenes such as ras, c-myc, and E1A stabilize p53 by inducing the expression of p14, the product of the alternate reading frame (ARF) of the INK4A locus (Sherr, 1998). p14 binds to the MDM2 protein and blocks mdm2-mediated degradation of p53 (Kamijo,

1998). Two models have been proposed to explain how p14 blocks the degradation of p53 by mdm2. One model proposes that p14 prevents the export of the p53/mdm2 complex to the cytoplasm (Kamijo, 1998) while the other proposes that p14 inhibits the ubiquitination of p53 by mdm2 (Honda, 1999). Although over-expression of p14 leads to accumulation of p53 and to p53-mediated transactivation, p14 does not activate the latent DNA binding capability of p53 in an *in vitro* DNA binding assay (Kamijo, 1998). This suggests that p14 can cause the accumulation of p53 but not its activation.

How other types of stress stabilize and activate p53 is less clear. Under low oxygen conditions, the association of p53 with the re-dox-sensitive protein HIF-1 alpha might play a role in activating p53 (An, 1998). Another redox-sensitive protein, Ref-1, has been shown to enhance the sequence-specific DNA binding activity of p53 (Jayarama, 1997). DNA damaging agents such as camptothecin and etoposide have been shown to inhibit transcription of the mdm2 gene (Xiao, 2000, Arriolla, 1999) and this may contribute to the stabilization of p53. These drugs have also been shown to induce phosphorylation of p53 at the amino terminus (Knippschild, 1997; Xiao, 2000) but the significance of the phosphorylation is still unclear.

The p53 response

The p53 response to stress includes but is not limited to cell cycle arrest and apoptosis. Why p53 executes one or the other is not clear but the decision seems to be influenced by genetic background (Polka, 1996), the extent of DNA damage (Chen, 1996) and/or cell type: apoptosis is more readily induced in cells of hematopoetic origin (Agarwal, 1998).

Growth Arrest

p53 can induce both G1 and G2/M cell cycle arrest. The p53-dependent G1 arrest is mediated by the cyclin kinase inhibitor p21 (Agarwal, 1998). This became evident with the demonstration that fibroblasts from p21-/- mice are deficient in their ability to growth arrest in response to DNA damage (Deng, 1995). Activated p53 binds to and enhances transcription of the p21 gene (El-deiry, 1993). The product of the p21 gene binds to G1-specific cyclin-dependent kinase complexes and blocks their kinase activity (Harper, 1993) which is needed for progression through S-phase. While the p53-dependent G1 cell cycle arrest is solely mediated by p21, the p53-dependent G2/M arrest is mediated by more than one of the p53 target genes. Activated p53 binds to and activates transcription of both 14-3-3σ (Hermeking, 1997) and the Gadd45 (Fornace, 1993) genes. The products of these genes play a role in the arrest of cells at the G2/M border (Wang, 1999). 14-3-3σ causes G2/M arrest by binding and sequestering cdc25c: a phosphotase whose activity is required for the activation of the G2/M cyclin dependent kinase: cyclinB/cdc2 (Kumagai, 1999 and 1998). GADD45 has been proposed to induce G2/M arrest by binding to and inhibiting cyclin B/cdc2 (Zhan, 1999).

Apoptosis

While p53-dependent cell cycle arrest is dependent on p53's transcriptional activity, p53-dependent apoptosis can occur in the absence of transcription (Bates, 1999). p53-dependent apoptosis can be induced in the presence of transcriptional and translational inhibitors (Caelles, 1994) and by the transcriptionally deficient mutant p53 gln22-ser23 (Haupt, 1995). This indicates that p53 transcriptional activity is not required for p53-dependent apoptosis. The XPB and XPD DNA helicases might play a role in the transcriptional-independent apoptosis induced by p53 as suggested by the observation that fibroblasts deficient for either XPB or XPD are defective in p53-mediated apoptosis (Wang, 1996). Re-introduction of either XPB or XPD induces p53-dependent apoptosis of these cells that is independent of the transcriptional activity of p53 (Wang, 1996). Although it has been shown that p53 can bind to and inhibit the DNA helicase activity of XPD and XPD, it remains unclear how this would trigger apoptosis (Wang, 1995).

p53, however, can activate transcription of a number of genes that play important roles in the apoptotic pathway (El-diery, 1998). p53 has been shown to activate transcription of the pro-apoptotic genes bax (Miyashita, 1995) and IGF-BP3 (Buckbinder, 1995). Transcription of the potent apoptotic inducer genes Fas/ApoI (Owen-Scaub, 1995) and the death domain containing receptor DR5 is also enhanced by p53 (Wu, 1997). The products of these genes trigger apoptosis by acting at different stages of the apoptotic pathway. BAX has been shown to trigger the release of cytochrome c from the mitochondria into the cytosol; an event that leads to activation of caspasces (Rosse,

1998). IGF-BP3 is a protein that binds to and inhibits the activity of the survival factor Insulin-like Growth Factor (IGF) (Hsu, 1992). Both FAS/APO and DR5 are death-domain-containing receptors whose engagement leads to the activation of caspases (Ashkenazi, 1998). p53 therefore can induce apoptosis in both a transcriptional-dependent and independent manner.

There is also evidence that transcriptional repression by p53 plays a role in the induction of apoptosis. Two proteins that inhibit p53-mediated apoptosis, the adenovirus E1B 19-kD protein and the cellular protein Bcl-2, have been reported to block p53's transcriptional repression activity but to have no effect on p53's transcriptional activation activity (Sabbatini, 1995; Shen, 1994). The product of the Wilm's tumor suppressor gene WT1 has also been shown to inhibit repression of transcription by p53 and to block p53-mediated apoptosis without affecting the ability of p53 to activate transcription (Maheswaran, 1995). It is not clear, however, whether repression of transcription causes apoptosis or repression of transcription is the result of apoptosis (Ryan, 1996).

Terminating the Response

Once the insult to DNA has been repaired, the p53 response must be terminated. In addition to the fact that the triggering stress is no longer present, p53 terminates its own response by a complex negative-regulatory loop that involves the mdm2 gene product (Oren, 1999). Once p53 is activated, it binds to and activates transcription of the mdm2

gene(Barak, 1993; Juven, 1993) whose product promotes the proteolytic degradation of p53 (Haupt, 1997, Kubbutat, 1997). It is unclear how this works mechanistically for p53 must also activate transcription of the genes that mediate its response. The observation that in some cases mdm2 is induced later than the other p53 responsive genes partially helps to explain this complex loop. This delay in mdm2 induction would give p53 time to carry out its responses (Yu, 2000).

Mutants of p53

A gene of such importance for maintaining genomic integrity is likely to be inactivated during the process of tumorigenesis. In fact, mutations of p53 are the most common aberrations detected in human tumors (Greenblatt, 1994). Sequencing of the p53 gene in more than 2100 tumors has revealed a number of unique properties of the mutations. Unlike other tumor suppressors, more than 90% of p53 mutations are missense rather than deletions or nonsense mutations (Hollstein, 1994). Furthermore, the mutations are clustered around the central sequence-specific DNA binding domain: preferentially targeting amino acid residues that are critical for DNA binding (Prives, 1994). In fact, more than 40% of the mutations preferentially alter amino acid residues R175, G245, R248, R249, R273 and R282 (Hollstein, 1994). Two explanations have been put forward to explain this pattern of mutations and they need not to be mutually exclusive. One is that the sequence-specific DNA binding activity of p53 is crucial for p53's tumor suppressor function. The fact that the most common alterations are to amino acid residues that are important for DNA binding support this notion. Of the six "hot

spot" mutants, R273 makes crucial contacts with the phosphate backbone in the major grove and R248 anchors p53 to the DNA minor grove with four hydrogen bonds (Cho, 1994). However, deletions or nonsense mutations could also disrupt the DNA binding activity of p53. In fact, deletions or nonsense mutations could be more effective in inactivating the DNA binding activity of p53. This can be best illustrated by the fact that some of the missense mutations can be re-activated to bind to DNA by many factors (Selivanova, 1998 and below). Since deletions and nonsense mutations would lead to the total or partial deletion of the DNA binding domain, there would be no possibility of re-activation of DNA binding.

An alternative explanation is that this pattern of mutations are found in tumors because they are selected due to their positive contribution to tumorigenesis. In fact, p53 was originally identified as an oncogene because of its ability to immortalize primary cells (Jenkins, 1984), to cooperate with ras in transforming primary cells (Eliyahu, 1984; Parada, 1984), and to give a tumorigenic phenotype to established cell lines (Eliyahu, 1985). When later it was determined that these activities were due to mutations in p53, it was concluded that mutant p53 has oncogenic properties (Hinds, 1989) and that the wild type protein is a tumor suppressor (Finlay, 1989). Mutant p53 can contribute to tumorigenesis by a transdominant inhibition of the wild type protein (Harvey, 1995; Kern, 1992; Zhang, 1993; Unger, 1992; Hachiya, 1994). Wt p53 binds to DNA as a tetramer –dimer of dimers- so it is easy to envision how associating with a non-DNA binding partner would alter the ability of the wt protein to bind to DNA (Herskowitz, 1987). In experimental systems, however, the transdominant effect is only observed

after over-expression of the mutant over the wt protein (Harvey, 1995; Zhang, 1993; Unger, 1992). When the wt and mutant protein are expressed from bi-directional vectors that allow for equal transcription of the wild type and mutant gene, the effect is only partial (Aurelio, 2000) as it is in fibroblasts of Li-Fraumani patients who inherit a mutant p53 allele (Srivastava, 1993).

Although the immortalization and transformation properties of mutant p53 were explained as a dominant-negative effect of the mutant over the wild type protein, additional experiments in p53 null cells have revealed the gain of function activities of mutant p53 (Dittmer, 1993). It has been shown that tumor-derived mutants of p53 can enhance the proliferation rate and tumorigenecity of p53 negative cells (Dittmer, 1993). These activities of mutant p53 require the activation domain (Lin, 1995) and the oligomererization/nonsequence-specific DNA binding domain (Lanyi, 1998).

Conformation Vs contact mutants

A number of studies have indicated that all mutants of p53 are not alike. By analyzing the DNA-bound crystal structure of p53, the mutants have been classified into two classes (Cho, 1996). Type I mutations are those in which the amino acid residues that make crucial contact with DNA are changed and therefore referred as "contact mutants". Type II mutations are the mutations that affect the proper folding of the protein and are therefore referred to as "conformational mutants". The type II mutants

share a number of properties that are not found in the type I mutants. Some of these properties include reactivity to p53-specific antibody 240 (Gannon, 1990), ability to bind to hs70 (Finlay, 1988) and resistance to degradation by calpains (Pariat, 1997). Additionally, unlike wt p53 which gives a 27kD thermolysin-resistant fragment, most conformational mutants are completely degraded by treatment with thermolysin (Bargonetti, 1993). Contact mutants such as His 273 behave like wt p53 in that they generate a thermolysin resistant fragment (Bargonetti, 1993), they are not reactive to antibody 240 (Gannon, 1990), appear not to bind to hs70 (Hinds, 1990), and are sensitive to degradation by calpains (Pariat, 1997).

Site-selective DNA binding mutants

Although mutants of p53 are generally characterized for their inability to bind to DNA, some tumor derived mutants (175P, 181L, 143A) have selective sequence-specific DNA binding and transcriptional activity (Ludwig, 1996; Ryan, 1998; Friedlander, 1996). These discriminatory mutants, although able to cause cell cycle arrest, can not induce apoptosis (Ludwig, 1996; Ryan, 1998; Friedlander, 1996). This is consistent with the observation that these mutants can activate transcription of the cyclin dependent kinase inhibitor p21 gene but not the apoptotic inducers Bax and IGF-BP3 (Ludwig, 1996; Ryan, 1998; Friedlander, 1996). Although, it has recently been reported that these mutants can activate transcription of the Fas/Apo-1 death receptor gene (Munsch, 2000). Importantly, the ability of these tumor-derived mutants to activate transcription was detected only when transfected into p53-/- cells in reporter gene assays. It is unclear

whether these mutants can launch a stress response like the wild type protein. The fact that these mutants are found in tumors suggests that they can't trigger a stress response or that the cells from which these mutants are derived have mutations in the p53 signaling pathway. Alternatively, these data suggest that the apoptotic activity of p53 is more important for its tumor suppressor function than the cell cycle arrest activity. The latter is consistent with the observation that these discriminatory mutants can't inhibit oncogene-mediated transformation (Crook, 1994).

Re-activation of mutant p53 transactivation

There are also some tumor-derived mutants of p53 that can be re-activated to bind to DNA and to activate transcription (review by Salivanova, 1998). Some of the factors that can re-activate sequence-specific DNA binding of mutant p53 include changes in temperature (Friedlander, 1996), binding of peptide derived from the carboxyl terminus of p53 (Abarzua, 1996; Salivanova, 1997), binding of the monoclonal antibody 421(Hupp, 1995) and small synthetic molecules (Foster, 1999). It is interesting to note however that although these factors can re-activate sequence-specific DNA binding of many mutants of p53, the transcriptional activity is only restored to a few. The carboxyl terminal peptides and monoclonal antibody PAb421 can only restore the transcriptional activity of the mutant p53 His 273. Furthermore, this mutant can only be reactivated to bind to and activate transcription from the synthetic non-genomic super consensus sequence (Abarzua, 1996; Hupp, 1993). The only temperature-sensitive, tumor-derived mutant is p53Ala143, but this mutant can bind to DNA and activate transcription from

only some of the wt p53 responsive genes (Friedlander, 1996). The small synthetic molecules not only re-activate the transactivation activity of some mutants of p53 (mainly His 273) but also slow the growth in nude mice of small human tumor xenografts with naturally mutated p53 (Foster, 1999). The inability of the rest of the mutants to activate transcription may be explained by their severe conformational changes. The mutants that are able to activate transcription upon reactivation of sequence-specific DNA binding appear to be those that can still associate with basal transcriptional machinery (Truant, 1993). Interestingly, these are the same mutants that when fused to a GAL4 DNA binding domain can activate transcription of reporter genes driven by Gal4 binding sites (Raycroft, 1991).

Stability of mutant p53

Another characteristic shared by most, if not all, mutants of p53 is an unusual accumulation of these proteins (Review by Save, 1998). The higher level of protein is not due to an increase in synthesis of the protein but rather to a decrease in degradation (Review by Blagosklonny, 1997). The decreased degradation was originally attributed to the altered conformation of the mutant proteins (Finlay, 1988). The conformation model, however, does not explain why mutants that retain a wild type conformation can also be expressed at high levels and why the wild type protein can be stabilized following a variety of stimuli. This lead to the proposal that the stabilization of mutant p53 is a result of the loss of the wt activity and, as a consequence, the loss of the negative regulatory loop (Review by Blagosklonny, 1997). Evidence for this model came from the demonstration that mutants of p53 were not expressed at high levels in cells also

expressing a functional p53 (Midgley, 1997; Hengstermann, 1998; Nagata, 1999). Although this model explains the reason for over-expression of most mutants, it does not account for the over-expression of both wild type and mutant p53 in cells that also over-express mdm2 (Landers, 1997). In cells that over-express mdm2, the high level of p53 appears to be due to either the inability of p53 to bind JNK and/or mdm2 or to over-expression of p14ARF (Buschmann, 2000). P14ARF has been shown to block the ubiquitination of p53 by mdm2 (Honda, 1999). Still not clear is the accumulation of conformationally wild type mutant p53 in cells that over-express mdm2 but not p14ARF. Alterations in the mdm2 protein could account for the stability of these mutants.

Mechanism for mutant p53 "gain of function"

At least three mechanisms have been proposed for the gain of function of mutant p53. 1) Mutant p53 promotes genomic instability. 2) Mutant p53 inhibits the activity of p53 family members. 3) Mutant p53 activates transcription of a set of genes that are not affected by wild type p53 and whose products promote cell proliferation.

Genomic instability

Although controversial, mutants of p53 unable to activate transcription can also demonstrate gain of function by generating genetic instability. Normal human fibroblasts growth arrest with a 4N DNA content in response to spindle depolymerization agents

such as colcemid. In contrast, fibroblasts from Li-Fraumeni syndrome (LFS) patients, which inherit type II mutations in one of their p53 alleles, re-replicate their DNA in the absence of chromosomal segregation. This effect of mutant p53 did not require a functional activation domain and was specific for the conformational (type II) mutants (Gualberto, 1998). Re-replication of DNA without chromosomal segregation can lead to genetic instability through the generation of polyploid cells. Consistent with this, fibroblasts derived from LFS patients accumulate aneuploid cells when cultured in vitro (Bischoff, 1990). Furthermore, mammary tumors from mice expressing the p53 conformational mutant 172H transgene in conjunction with a transgene coding for either neu, IGF-1 or TGFβ are frequently aneuploid. In contrast, mammary tumors from mice expressing either neu, IGF-1 or TGFβ in the absence of mutant p53 are not aneuploid (Murphy, 2000). Yet, the role of mutant p53 in causing genetic instability is questionable. First, genetic instability is an early event in human tumors but mutation of p53 is a late event (Reviewed by Langauer, 1998). Second, if mutant p53 was the cause of the genetic instability in the transgenic mice, all the transgenic mice would be expected to be genetically unstable. Likewise, all the LFS fibroblasts would be expected to be aneuploid. Third, it has also been shown that both mouse and human $p53^{-/-}$ fibroblasts also re-replicate their DNA in the presence of colcemid (Di Leonardo, 1997). This instability, therefore, might be a result of the lack of p53 function and not a gain of function

Whether through generating genetic instability or not, mice engineered to express the 172H mutation in one of their alleles ($p53^{+/172H}$) developed tumors with higher

metastatic potential than p53 $^{-/-}$ mice (Liu, 2000). Furthermore, unlike p53 $^{-/-}$ mice which readily undergo loss of heterozygosity, the p53 $^{-/172H}$ mice rarely lose the wild type allele (Liu, 2000).

A number of reported activities of mutant p53 may contribute to this genetic instability. Class II conformational mutants of p53 have been shown to promote intrachromosomal homologous recombination (Saintigny, 1999). Aberrant recombination events can lead to rearrangements such as inversions, deletions and duplication: all of which can promote genetic instability (Saintigny, 1999). Centrosome abnormalities such as the presence of more than two centrosomes per cell can lead to polyploidy. This type of centrosome abnormality was found with high frequency in transgenic mice expressing the172H class II mutant (Wang, 1998). Another activity of mutant p53 that may contribute to this genetic instability is the ability of mutant p53 to disrupt high order chromatin structure (Smith, 1998). This activity may be mediated by the ability of mutant p53 to bind with high affinity to Matrix Attachment Regions (MAR) DNA elements (Deppert, 1996). MAR DNA elements modulate important cellular processes such as gene expression, replication and recombination (Review by Deppert, 1996). Interestingly, this activity is present in both types of mutants (class I and II). It can be envisioned that binding of the type I mutants could lead to aberrant gene expression while binding of the class II mutants would lead to aberrant recombination. Finally mutant p53 could alter recombination not only by binding to MAR elements but also by associating and stimulating the activity of topoisomerase I (Albor, 1998).

The case of p73 and p63

Re-interpretation of the gain of function of mutant p53 has been made necessary by the identification of p53 family members. p73 and p63 have been recently cloned and shown to share extensive homology to p53, particularly in the DNA binding domain, the amino-terminus transactivation domain and the oligomerization domain (Review by Kaelin, 199). As expected from the homology, both p73 and p63 bind to p53 binding sites in gel mobility shift assays, can activate transcription of p53 target genes and can induce apoptosis of cells when over-expressed (Levrero, 2000). Like p53, p73 can be activated by some of the same DNA damaging agents that activate p53 (Lohrum, 2000). Unlike p53, however, mutations of p73 are rare and mice deficient in p73 or p63 are not tumor-prone like mice lacking p53 (Review by Soengas, 2000). Unlike p53, which is commonly inactivated by viral oncoproteins, p73 is not affected (Marin, 1998). The function of p73, like p53, is inhibited by the MDM2 protein but MDM2 does not promote degradation of p73 as is the case for p53 (Zeng, 1999) Despite the extensive homology and some functional redundancy, therefore, some differences are beginning to emerge among p53 family members.

The homology in the oligomerization domain among p53 family members prompted the question of possible dominant-negative inhibition of p73 or p63 by mutant p53. If mutant p53 could inhibit the ability of p73 and p63 to induce cell cycle arrest and apoptosis, this could explain the ability of tumor-derived mutants of p53 to enhance the

proliferation rate and tumorigenecity of p53 negative cells (Dittmer, 1993). Conflicting results have been published on this matter. Davison et al. (1999) showed that the oligomerization domain of p53 does not associate with that of either p73 or p63 and that the type I (contact) p53 mutant His 273 did not inhibit in a dominant-negative manner the ability of p73 to activate transcription of a reporter gene. Di Como et al (1999) on the other hand, showed that p73 co-immunoprecipitated with mutant p53 but not with wt p53 and that mutant p53 R175H and R248W inhibited the transactivation activity of p73 and more importantly, the ability of p73 to induce apoptosis. These opposite results have been partially explained by a recent report showing that the association is not through the oligomerization domain. The same study also showed that p73 prefers to associate with the type II (conformational) mutant p53 that codes for argenine, instead of proline, at the polymorphic codon 72 of p53 (Marin, 2000). These data suggest that some conformational mutants of p53 can inhibit the function of p73. An extension of this correlation is the observation that the mutants of p53 that can block the killing of p53 defective cells with etoposide (Blandino, 1999) are those that were shown to associate with p73 (Di Como, 1999; Marin, 2000). In some cases, therefore, the dominant negative effect of mutant p53 on p73 could account for the gain of function activity of mutant p53.

Activation of transcription by mutant p53

The "gain of function" activity of mutant p53 requires the amino terminal transactivation domain (Lin, 1995). This suggested that transactivation of growth promoting genes could be the mechanism by which some mutants of p53 give cells a

growth advantage. Consistent with this, a number of genes have been shown to be transactivated by mutants of p53. These tumor-derived p53 mutants can activate transcription of cellular promoters such as the Epidermal Grow Factor Receptor promoter (Ludes-Meyers, 1996), the Proliferating Cell Nuclear Antigen promoter (Deb, 1992), the multi drug resistant gene (MDR-1) promoter (Chin 1992), the c-myc gene (Frazier, 1998), the promoter of the anti-apoptotic gene BAG-1 (Yang, 1999), and a number of genes from the Ribosomal Gene Cluster (Loging, 1999) and the HIV-LTR (Subler, 1994). It remains unclear how mutants of p53 activate transcription of these promoters.

We have used the HIV-LTR to studied the mechanism of mutant p53 transactivation. The long terminal repeat (LTR) of the human immunodeficiency virus (HIV) regulates transcription of the human immunodeficiency virus (HIV) proviral genes by binding many viral and cellular transcription factors (Cullen, 1989). The HIV-LTR is organized into four functional regions with respect to the location of cis-acting DNA regulatory elements (Pereira, 2000). The transactivation response element (TAR) region, defined for its requirement for Tat-induced transactivation, has been shown to bind other transcription factors (Cullen, 1986). Some of these factors can activate while other can repress transcription (Pereira, 2000). The basal or core promoter region can bind more than 16 cellular factors in addition to the TATA binding protein (TBP) and the ubiquitous transcription factor Sp1. The enhancer or modulatory region can bind more than 40 cellular factors in addition to the NFkB/Rel family members (Pereira, 2000). Many of the factors that bind to the distal enhancer region are particularly important for the

transcription of the chromatin-bound-genomic-integrated HIV proviral genes (Kim, 1993).

Many studies have shown that the three Sp1 binding sites in the core promoter region play a key role in the regulation of HIV transcription. Deletion of these Sp1 binding sites decreases both basal and activated gene expression (Garcia, 1987). This decrease in transcription is not only due to the lack of DNA binding by Sp1. DNAse footprinting analysis have shown that the Sp1 binding sites regulate the binding of other factors to flanking regions in the HIV-LTR (Harrich, 1989). Consistent with this observation, the Sp1 binding sites region is required for NF-kB induced transcription in reporter assays (Perkins, 1993) and in chromatin templates (Pazin, 1996) indicating that the binding of Sp1 facilitates NFkB binding. The Sp1 binding site region is also required for Tat-induced transcription (Garcia, 1988) indicating a requirement for Sp1 DNA binding in facilitating the binding of Tat. These observations predict an association between NFkB and Tat with Sp1. In fact, both NFkB and Tat have been shown to associate with Sp1(Jeang, 1993; Perkins, 1993).

Sp1 is a Zinc finger DNA binding transcription factor that binds to GC rich regions called GC Boxes (Naar, 1998). The glutamine rich activation domains at the N-terminus of Sp1 interact with members of the general transcription machinery (Gill, 1994). These activation domains are essential for transcriptional activation (Courey, 1989) and for synergistic activation when Sp1 binds to more than one site (Pascal, 1991). The association of Sp1 with other transcription factors is also essential for synergistic

activation (Li, 1991; Pazin, 1996; Garcia, 1988; Sheridan, 1995). The Sp1 requirement for other factors to activate transcription may be due to the ability of Sp1 to bind nucleosome-bound DNA (Li, 1991) and to remodel chromatin structure (Jongstra, 1984; Sheridan, 1995). In fact, transcription factors such as LEF-1, Ets-1, and TFE-3 can not activate transcription of nucleosome-assembled DNA in the absence of Sp1 (Sheridan, 1995). Sp1, therefore, is involved in synergistic activation by associating with other Sp1 molecules bound at other sites and by opening the chromatin structure and facilitating the binding of other transcription factors to their corresponding binding sites in the HIV-LTR.

Deletion analysis of the HIV-LTR have revealed that the three Sp1 binding sites of the HIV-LTR are important for mutant p53 transactivation (Subler, 1994). We have previously shown that purified mutant p53 does not bind to these three Sp1 binding sites (Bargonetti, 1997). Since Sp1 has been shown to associate with other transcription factor to activate transcription (Kardassis, 1999), we proposed and tested a model whereby mutants of p53 activate transcription by associating with DNA bound Sp1 (Fig. 1.1). Using DNA affinity chromatography, we showed that mutant p53 His 273 in the MDA 468 cells formed a complex with Sp1 on HIV-LTR DNA. This complex, which is not detected by co-immunoprecipitation, is also formed on the p53 super consensus sequence (SCS). We showed that this endogenously expressed mutant p53 his 273, like transfected mutant p53, can activate HIV-LTR driven transcription (Chapter, 3). These data are consistent with our model. Together with previous results, these data suggest that the gain of function activity of mutant p53 may come from the ability of mutant p53 to associate with DNA bound Sp1. This association could lead to an increase in the

expression of genes containing Sp1 binding sites. Interestingly, Sp1 binding sites are commonly found in housekeeping and growth promoting genes (Naar, 1998).

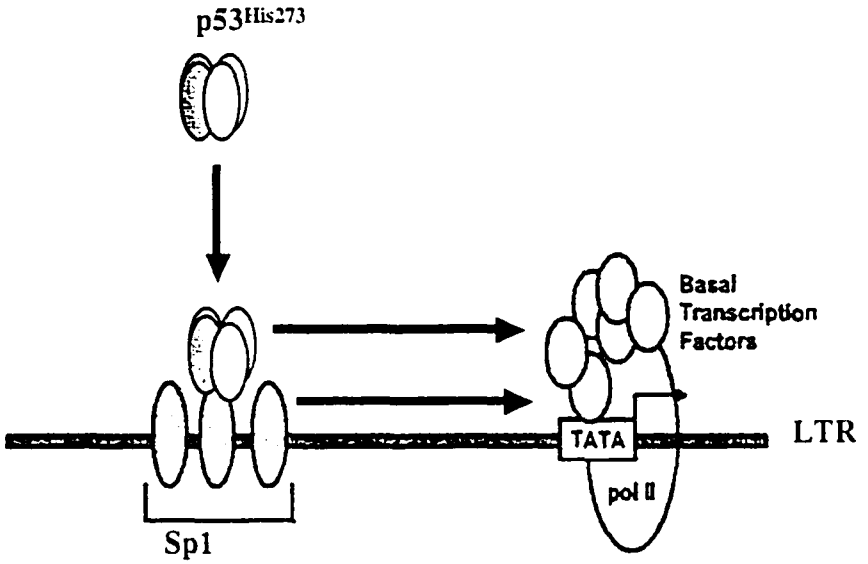


Figure 1.1 **Model of mutant p53 transactivation from the HIV-LTR.** Mutant p53 associates with Sp1 bound to its binding sites. This association could lead to the stabilization of the pre-initiation complex and therefore to an increase in HIV-LTR driven transcription.

Chapter 2

Materials and methods

Cells and viruses

The MDAH041 line is a human fibroblast cell line lacking functional p53 protein due to a frameshift mutation of one p53 allele at codon 184 and loss of the normal p53 allele (Agarwal. 1995). The TR9-7 cell line is an isogenic line derived from MDAH041 that contains a tetracycline regulated wild-type p53 (generously provided by Dr. Agarwal. (Agarwal. 1995). The MDA-MB-468 cell line is a tumor-derived line that expresses mutant p53 His 273 (Nigro. 1989). This cell line was obtained from American Type Culture Collection. MDAH041. TR9-7 and MDA-MB-468 cells were grown in DMEM media supplemented with 10% heat inactivated fetal bovine serum under 5% CO_2. TR9-7 cells were grown in the presence of 2ug/ml Tetracycline. 0.6mg/mL Neomycin and 50ug/mL Hygromycin. p53 induction was achieved after 24 hours of removal of Tetracycline. Spodoptera frugiperda cells (Sf21 cells) and recombinant baculoviruses expressing wild-type human p53 were as described (O'Reilly and Miller. 1988). Sf21 cells (2.5×10^7 / 150 mm dish) were infected with recombinant viruses and harvested 48 hr post infection. Extracts of infected cells were prepared as described (Bargonetti. 1992).

Oligonucleotides and plasmids

All the oligonucleotides were purchased from Operon Technologies (Alamerica. CA). The HIV oligonucleotide contains the sequence: 5'GGATCCGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGG CGA-3', which includes the three Sp1 binding sites of the HIV long terminal repeat. The

SCS synthetic oligonucleotide contains the p53 consensus binding sites (Halazonetis, 1993) as follows:

5' TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC - 3'. The murine mdm2 synthetic oligonucleotide used in this study contains the two p53 response elements from the mdm2 gene. The sequence of this oligonucleotide is as follows:

5' - GATCCCTGGTCAAGTTGGGACACGTCCGGCGTCGGCTGTCGGAGGAGC TAAGTCCTGACATGTCTCCG - 3'. The oligonucleotide mutated for wild type p53 binding is derived from the Ribosomal Gene Cluster and contains the sequence:

5'-TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC-3'. The luciferase expression reporter (pLTRLuc) was constructed by inserting into the Xho1-HindIII sites of the pGL2 basic vector the Xho1-HindIII digested fragment from the pLTRCAT.

Transfection and Luciferase Assays.

Cells were harvested at 80% confluence, washed twice with serum free DMEM and resuspended to 1 x 10^7 cells per milliliter. 500uL of resuspended cells were mixed in Gene Pulser Cuvettes (Bio-Rad) with the indicated plasmid DNA, incubated on ice for 10 minutes and electroporated using Gene PulserR (Bio-Rad) at 350V. The cells were incubated on ice for 10 minutes and plated on 100 mm plates with DMEM plus 10% FBS and then placed at 37^0C. After 48 hours, cells were harvested and cell extracts were prepared using Reporter Lysis buffer (Promega) according to the manufacturer's instructions. Where indicated, transfected cells were treated with EGF (100ng/mL) for 24, 12, 9, 6, or 3 hours before lysis. PKC inhibitors were added 30 minutes before EGF

treatment where indicated . GF1092X was used at a concentration of 5μM and Rottlerin was used at a concentration of 1μM. Luciferase assays were performed with 20ul of extracts and 100uL of substrate in a scintillation counter in the manual mode. Luciferase activity was normalized for β-galactosidase activity for the transactivation studies and for μg of total protein for the repression studies. The relative luciferase activity represents the average activity of three separate transfections.

Preparation of nuclear protein extracts

Two methods were used to prepare cell extracts. Method 1 was used to prepare nuclear extracts from MDA-MB-468 cells for DNA affinity chromatogragy. After washing twice with cold 1xPBS, the cells were scraped and pelleted by centrifugation for 10 minutes at 3000 rpm on a Beckman centrifuge. The pellet was resuspended in 5 packed cell volumes of hypotonic buffer (10 mM Hepes, pH 7.9 at 4 C; 1.5mM MgCL; 10mM KCL; 0.2mM PMSF; 0.5mM EDTA) and quickly pelleted by spinning as above for 5 minutes. The cells were swelled for 10 minutes in 3 packed cell volumes of hypotonic buffer, homogenized in a glass dounce homogenizer with 10 strokes using a type B pestle, and spun for 15 minutes at 3800 rpm. The pellet containing the nuclei was re-suspended in 0.5 of the packed nuclei volume with low salt buffer (20 mM hepes, pH 7.9; 25% glycerol, 1.5 mM MgCL, 0.02M KCL, 0.2 mM EDTA, 0.2 mM PMSF, 0.5mM DTT) and 0.5 of the packed nuclei volume of high salt buffer (same as low salt buffer except that KCL concentration was 1.2M). The re-suspended pellet was rocked for 30 minutes and the nuclear proteins were obtained by centrifugation for 30 minutes at 13000 rpm in a microcentrifuge.

Method 2 was used to prepare nuclear lysates from 041 and TR9-7 cells for DNA affinity chromatography as well as to prepare extracts for immunoprecipitations and Western blots of MDA-MB-468, TR9-7 and 041 cells. The cells were washed two times with 4^0C 1x PBS. Cytoplasmic Lysis Buffer was prepared with 8.8 ml of Lysis Buffer Stock (20mM Hepes pH 7.5, 20% Glycerol, 10mM NaCl, 1.5mM MgCl2, 0.2 mM EDTA pH 8.0, 0.1% Triton X-100, 1 mM DTT, 1mM PMSF, 50 ug/ml aprotinin, 50uM leupeptin) and 6.2 ml of ddH_2O. One milliliter of Cytoplasmic Lysis Buffer was added to each 150 mm plate. The cells were scraped off the plate and spun at 2300 rpm at 4^0C for 10 minutes. The supernatant was removed and the pellet was resuspended in 0.1 ml of nuclear extraction buffer (prepared with 8.8 ml of Lysis Buffer Stock, 0.5 M NaCl, and 4.7 ml of dH2O). The cells were rocked with the nuclear extraction buffer for 1 hour at 4^0C and then centrifuged at 14000 rpm for 10 minutes to extract the nuclear proteins. Protein concentrations of the extracts were determined via Bradford Microassay (Bio-Rad).

DNA Affinity Chromatography.

The oligonucleotides used for DNA affinity chromatography were as described above. The DNA affinity resins were prepared as described (Kodanega, 1991). Briefly, complementary synthetic oligodeoxynucleotides were annealed and ligated to give at least 10-mers. The oligomers were covalently coupled to agarose beads with cyanogen bromide to yield the affinity resin. Nuclear extracts prepared as indicated above were fractionated on a Sephacryl S-300 column. The fractions were pooled and concentrated using Centriprep filters (Millipores). 1.5mg of protein from the Sephacryl S-300 pool

was incubated with nonspecific competitor DNA before passing it 10 times through the DNA columns. The columns were washed 5 times with 1.5mL of buffer Z (25 mM Hepes, pH 7.6; 0.1M KCL, 12.5 mM $MgCL_2$, 20% Glycerol, 0.1% IPEGAL, 1mM DTT) and the bound factors were eluted with buffer Z containing increasing concentrations of KCL (0.2M to 1.0M) in 1 ml aliquots. The 1mL fractions were concentrated to 0.1mL.

Immunoprecipitation.

250 ug of nuclear protein from MDA 468 cells were incubated overnight at 4^0C with 1 ug of Sp1 antibody PEP2G (Santa Cruz, CA) in 500uL of Immunoprecipitation buffer (nuclear extraction buffer used in method 2 plus 0.2 mM sodium orthovenadate). 50 ul of protein-G-PLUS agarose (Santa Cruz, CA) were added and the mixture incubated for an additional 5 hours. The precipitates were washed 5 times with the IP buffer, boiled in protein sample buffer for 5 minutes and loaded on a 10 % SDS-polyacrylamide gel.

Western blot analysis.

Protein samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electrotransfered to a nitrocellulose membrane. The membranes were probed with the corresponding substrate-specific antibodies followed by incubation with the appropriate Horse Radish Peroxidase-conjugated antibody. Visualization was with ECL solutions (Amersham, Life Science).

EMSA

Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase and [32P] dCTP. Reaction mixtures were carried out in 30uL of a buffer containing 2ug of nuclear protein or 5% of elution fractions, 0.15 pmoles of radiolable oligonucleotide, 20 mM HEPES (pH 7.8), 100 mM KCl, 1 mM EDTA, 1mM DTT, 1.0 µg sheared salmon sperm DNA and 10% glycerol. 2ug of one appropriate antibody was added to each reaction where indicated. All samples were incubated at room temperature for 20 min. The protein-DNA complexes were resolved on a 4% acrylamide gel.

Chapter 3

Activation of HIV-LTR-driven transcription by Mutant p53

INTRODUCTION

Although some tumor-derived mutants of p53 can activate transcription of many growth promoting genes (Roemer, 1999), the mechanism of transactivation remains unknown. Wt p53 is a gene specific transactivator (Oren, 1999) but unlike wt p53, mutants of p53 are generally defective in sequence specific DNA binding (Zambetti, 1993). Although some p53 mutants can bind to DNA albeit differently than the wild type protein (Di Cuomo, 1998; Kern, 1991; Park, 1994; Niewolik, 1995), none of the mutants have been shown to bind to any of the genes that are transactivated by mutant p53. One of the promoters that is upregulated by mutant p53 is the HIV-LTR (Subler, 1994). The significance of the up-regulation was shown by the ability of mutant p53 to promote the replication of a dormant virus (Duan, 1994). We have used the HIV-LTR as a tool to examine the mechanism of transactivation by mutant p53.

A number of studies using the HIV-LTR suggested that mutant p53 proteins activate transcription by cooperating with the Sp1 transcription factor. First, the region of HIV-LTR containing the Sp1 binding sites is the minimal promoter element required for mutant p53 transactivation (Subler, 1994). Second, Sp1 has been shown to work together with other enhancer-binding and non-enhancer-binding transcription factors to synergistically activate the transcription of many different genes (Naar, 1998;Kardassis, 1999). Third, p53 has been shown to co-immunoprecipitate with Sp1. The association appears to require a mitogen-induced modification of p53 for it is only detected after treatment of cells with either Granulocyte Macrophage Colony Stimulating Factor

(Borellini, 1993) or Tumor Necrosis Factor (TNF-alpha) (Gualberto, 1995b). These factors appear to alter the conformation of p53 into a conformation that is recognized by p53 specific antibody 240 (Gualberto, 1995). This antibody recognizes p53 that is in a denatured conformation; a characteristic of most mutants of p53 (Gannon, 1990). In the case of TNFα, the mutant p53-Sp1 complex was suggested to mediate TNF alpha-induced transactivation of the HIV-LTR (Gualberto, 1995). Since tumor-derived mutants of p53 activate HIV-LTR directed transcription in the absence of mitogen stimulation (Subler, 1994b), the data suggest that such mutants have the ability to associate with Sp1 to activate transcription under normal growth conditions. Interestingly, no study examining the association between Sp1 and tumor derived mutants of p53 has been reported.

We have examined the interaction between Sp1 and the endogenously expressed "hot-spot" mutant p53 His 273 from the human breast cancer cell line MDA-MB-468. This mutant has the ability to bind to DNA albeit differently than wild-type p53 (Di Cuomo, 1998; Kern, 1991; Park, 1994; Niewolik, 1995) and maintains an active transcriptional activation domain (Raycroft, 1991). We have looked to see if both His 273 p53 and Sp1 can associate as a complex with the Sp1 binding sites of the HIV-LTR as well as associate with a p53 super consensus sequence (SCS). We have also inactivated mutant p53 His 273 in MDA 468 cells to determine the contribution that this mutant p53 makes to HIV-LTR-directed transcription in MDA 468 cells.

RESULTS

His 273 p53 co-elutes with Sp1 from a Sp1 DNA affinity

DNA affinity chromatography has been used to purify the Sp1 protein (Briggs, 1986). Assuming that the ability of mutant p53 to activate transcription of the HIV-LTR is due to a multi-protein complex involving p53, Sp1 and the DNA, these proteins would be expected to co-purify from a DNA affinity column containing Sp1 binding sites. We have therefore investigated the ability of His 273 mutant p53 from the Breast Cancer cell line MDA-MB-468 to co-elute with the ubiquitous transcription factor Sp1 by DNA affinity purification using the protocol described by Kodanaga (Kodanaga, 1991). An oligonucleotide containing the three Sp1 binding sites of the HIV-LTR was synthesized and coupled to sepharose beads. Sephacryl -excluded nuclear extract from the MDA-MB- 468 cells was passed through the DNA-beads column to purify the sequence-specific DNA binding proteins and other associated factors. The column was washed 5 times and the bound factors were eluted with a buffer containing increasing concentrations of KCL (0.2M-1.0M). Western blot analysis was used to identify the presence of Sp1 and p53 in the DNA affinity column fractions (Fig. 3.1A and 3.1B). No Sp1 was detected in the column flow-through, indicating that most of the Sp1 remained bound to the DNA (Fig. 3.1A, FT). Much of the p53 His 273 did not interact with the column and was found in the flow through (Fig. 3.1B, FT). Neither p53 nor Sp1 were detectable in wash number 5 (Fig. 3.1A and 3.1B, W5). The eluted fractions showed that Sp1 was released from the column in the 0.4M and 0.5M KCL fractions (Fig. 3.1A, 0.4

and 0.5). Mutant p53 His 273 was detected in the 0.4 M KCL fraction when the blot was over-exposed, thus indicating that a small amount of p53 co-eluted with Sp1 from the DNA column (Fig. 3.1B, 0.4). The presence of p53 in the 0.4M elution fraction was also detectable by electrophoretic mobility shift assay (EMSA) using the p53 super-consensus sequence (SCS) (Halazonetis, 1993) (Fig. 3.1C). As shown previously, His 273 p53 can bind to idealized p53 binding sites in the presence of p53 antibody PAb 421 (Zhang, 1993a; Prasad, 1997). The His273 p53 was detectable in the elution fractions by both techniques in repeated experiments.

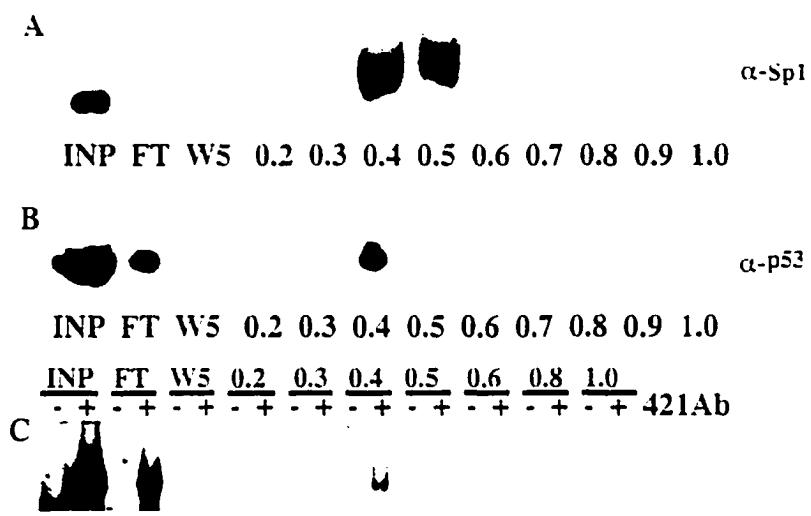


Fig. 3.1 **Mutant p53 and Sp1 from MDA 468 cells co-elute from the Sp1 column.** Western blot of 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) loaded where indicated. The blot was probed with an anti-Sp1 antibody (A) and with anti-p53 antibodies (B). C) EMSA using 5% of the fraction indicated and the p53 super consensus sequence (SCS) oligonucleotide in the presence (+) or absence (-) of the p53 specific antibody 421.

Wild type p53 does not associates with the Sp1-DNA column.

To examine if the association of p53 with the Sp1-DNA column was specific for mutant p53, we tested the ability of wild type p53 to associate with the Sp1-DNA column. For this purpose we used the TR9-7 cells. TR9-7 cells express wt p53 from a tetracycline regulated promoter in which removal of tetracycline induces high levels of wild type p53 (Agarwal, 1995). Sephacryl-excluded extract from the TR9-7 cells was passed through the Sp1-DNA column and the elution fractions tested for the presence of Sp1 and p53 by Western blot analysis. As with the MDA 468 cells, no Sp1 was detected in the column flow through indicating that most of the Sp1 remained bound to the DNA (Fig. 3.2A, FT). Wild type p53 did not interact with the column and was found in the flow through (Fig. 3.2B, FT). Neither p53 nor Sp1 were detectable in wash number 5 (Fig. 3.2A and 3.2B, W5). The eluted fractions showed that Sp1 was mainly released from the column in the 0.4M fraction and to a lesser extent in the 0.5M and 0.6M KCL fractions (Fig. 3.2A, 0.4, 0.5 and 0.6). Unlike mutant p53 His 273 which was detected in the 0.4 M KCL fraction, wild type p53 was not detected in any of the eluted fractions (Fig. 3.2B). We also examined the fractions for the presence of p53 by EMSA using the p53 super-consensus sequence(SCS). The binding of p53 was detected in the input lane and this binding was shifted with the p53-specific antibody 1801. No binding of wt p53 was detected in any of the elution fractions (Fig. 3.2C).

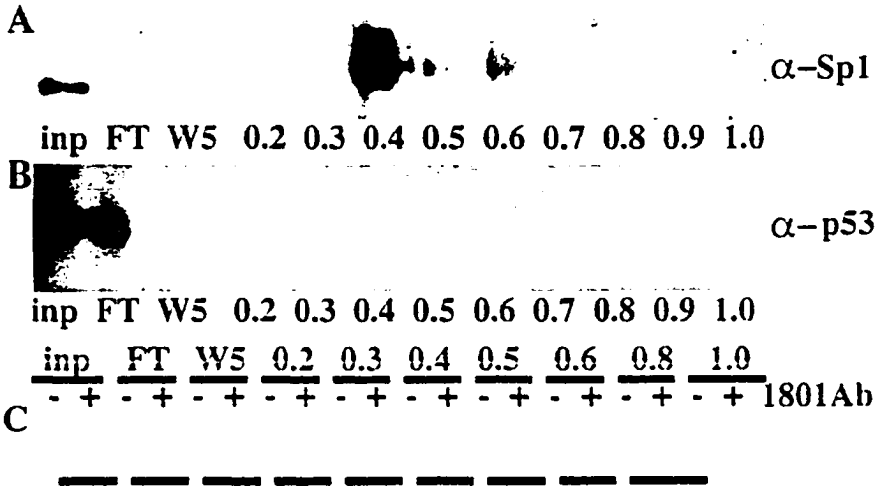


Figure 3.2. Wt p53 does not co-elute with Sp1 from Sp1 DNA column.
Western blot of 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) loaded where indicated. The blot was probed with anti-Sp1 antibody (A) and with anti-p53 antibodies (B). C) EMSA using 5% of the fraction indicated and the p53 super consensus sequence (SCS) oligonucleotide in the presence (+) or absence (-) of the p53 specific antibody 1801.

Sp1 co-elutes with mutant p53 from a SCS affinity column.

To further examine the ability of a MDA-MB-468 p53-Sp1 complex to interact with DNA, we utilized a DNA affinity column containing the p53 super consensus sequence (SCS). We first examined the ability of His 273 p53 present in MDA-MB-468 nuclear extract to bind sequence specifically to the SCS oligonucleotides by EMSA (Fig. 3.3A). Mutant p53 binding was detected in the presence of the p53 specific antibody 421(lane 2) and this binding was specific as it was only competed with an excess of un-

labeled SCS oligonucleotide (Fig. 3.3A, lanes 3 & 4). The DNA binding was not competed with an excess of either mutated p53 site oligonucleotide (Fig. 3.3A, lanes 5 & 6), mdm2 site oligonucleotide (lanes 7&8) or HIV-LTR Sp1 binding site oligonucleotide (lanes 9 &10). Note that although His 273 p53 was able to bind the SCS site, it did not bind to the mdm2 p53 binding site oligonucleotide or to the HIV-LTR sequence (Fig. 3.3A, compare lanes 3 & 4 to 7-10).

To determine if Sp1 could co-purify with the mutant p53 His 273 in the absence of a Sp1 binding site, nuclear extract from MDA-MB-468 cells was passed through a SCS oligonucleotide DNA-affinity column. The elution fractions were then examined for the presence of Sp1 and p53 by Western blot analysis (Figure 3B-C). Interestingly both Sp1 and p53 were detected in the 0.4M KCL elution fraction (see Fig 3.3B for Sp1 and 3.3C for p53). In clear contrast to the high affinity binding seen for Sp1 when the HIV-LTR column was used, this time Sp1 was detected in the flow through (compare FT in Fig. 3.1A to 3.3B). Much of the p53 was found in the flow-through, indicating that only a portion of the mutant p53 was competent for binding to DNA (Figure 3.3C; FT). His273 p53 was not detected in wash 5 indicating that the p53 in the elution fractions resulted from tightly associated p53. The p53 Western blot did not need to be over-exposed (as was required for the HIV-LTR column fractions) in order to see the p53 in the 0.4M KCl elution fraction, indicating that more p53 was associated with the SCS DNA affinity column than with the HIV-LTR affinity column. These data suggest that when Sp1 is bound to DNA it can also interact with a subset of cellular His 273 p53 by a protein-protein association and conversely, when His 273 p53 is bound to DNA it can also interact with Sp1 by a protein-protein association.

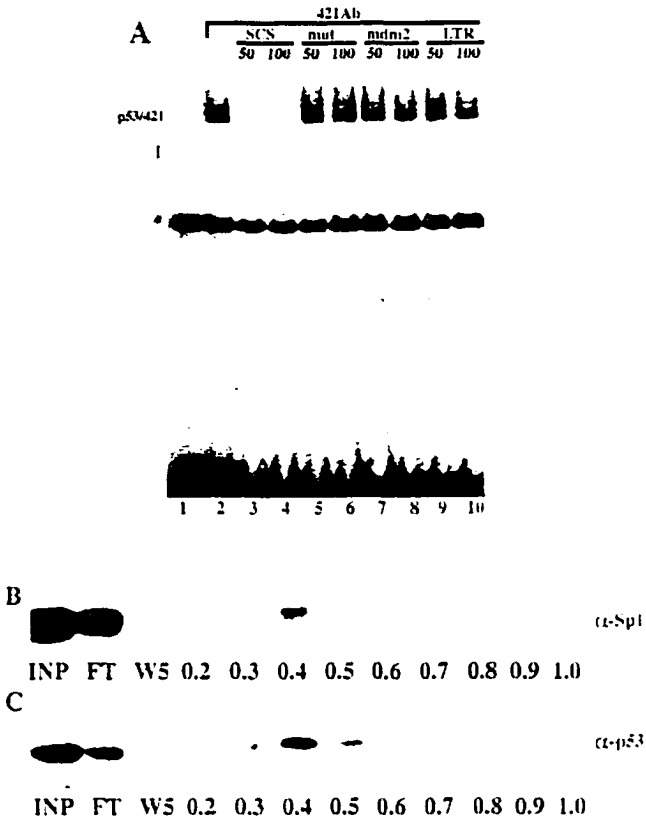


Fig.3.3A Sequence specific DNA binding of mutant p53 His 273 in MDA 468 cells to SCS. Binding was not detected in the absence of antibody (lane 1). The p53 antibody 421(2ug) induced the binding of p53 (lane 2). This binding was competed with cold excess SCS (lanes 3-4) but not with a site mutated for wt p53 binding (5-6), a site containing the p53 responsive elements of the mdm2 gene (7-8) or the oligo containing Sp1 binding sites (9-10). Competition was with 50 (3.5.7.9) or 100 fold (4.6.8.10) of the indicated oligonucleotide.

Fig.3.3B and 3.3C. Mutant p53 and Sp1 from MDA 468 cells co-elute from SCS column. MDA 468 extract was passed through DNA column made with the SCS oligonucleotide. 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were loaded where indicated. The blot was probed with anti-Sp1 antibody (B) and with anti-p53 antibodies (C).

Sp1 association with p53 binding sites requires the DNA binding activity of mutant p53

To rule out the possibility that Sp1 could directly bind to p53 binding sites under DNA affinity conditions, we passed nuclear extract from a cell line which does not express p53 (MDAH041) over the SCS column and analyzed the elution fractions by Western blot analysis for Sp1 (Fig. 3.4A). The Sp1 was found in the flow through and was not detected in any of the column fractions (Fig. 3.4A). This was a strong indication that the Sp1 eluting from the SCS column seen in Fig. 3.3B was not the result of a direct interaction but was recruited in some way by the His273 mutant p53. The MDAH041 extract was also passed over the HIV-LTR DNA affinity column to examine the DNA binding ability of the Sp1 in the absence of p53. The column fractions were analyzed by Western blot analysis. As in the MDA-MB-468 cells, Sp1 bound with high affinity as evident by the lack of Sp1 in the column flow through (Fig. 3.4B, FT). Additionally Sp1 was once again detected in the 0.4M and 0.5M fractions eluted from this HIV-LTR oligonucleotide column (Fig. 3.4B, 0.4 and 0.5).

To further address the ability of the Sp1 to interact with p53 binding sites only in the presence of His273 p53 DNA binding activity, we examined the ability of both Sp1 and His 273 p53 to elute from a mdm2 DNA affinity column (Figs. 3.4A & 3.4B). Nuclear extract of the MDA 468 cells was passed through a DNA column made with an oligonucleotide containing the p53 response element of the mdm2 gene. Western blot analysis of elution fractions from a mdm2 affinity column demonstrated that neither Sp1

protein nor His273 p53 could be isolated using this DNA sequence (Fig. 3.4C and 3.4D). His273 p53 does not bind to the mdm2 p53 binding site as shown by the inability of the mdm2 oligonucleotide to compete the binding of His 273 to SCS (Fig. 3.3A). Taken together, these results suggest that Sp1 can associate with DNA that does not have Sp1 binding sites via an interaction with His273 p53 when His273 is able to bind to DNA.

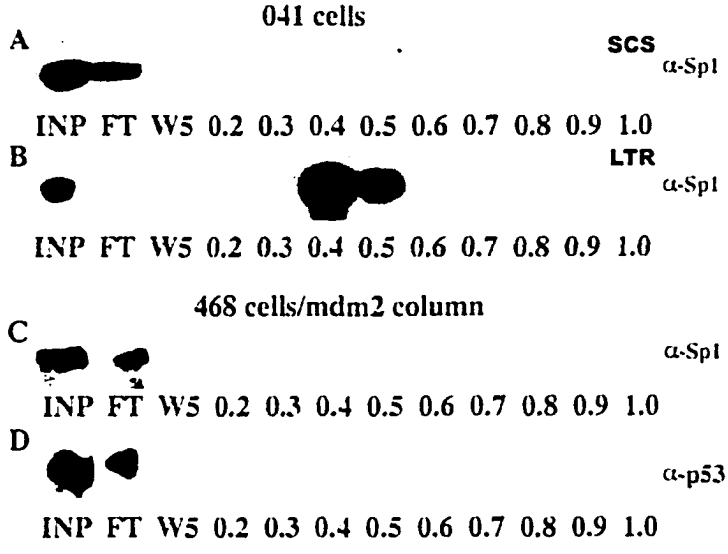
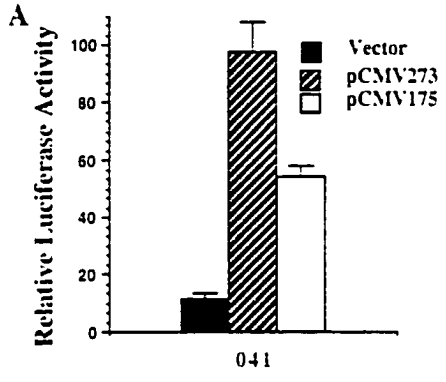


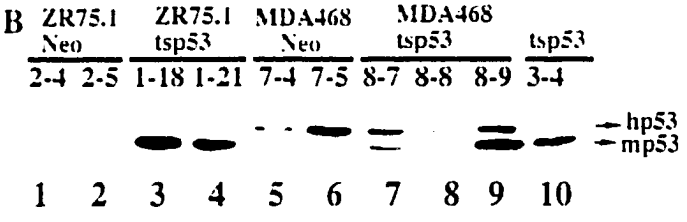
Fig.3.4. Sp1 does not associate with the SCS in the absence of p53.
p53 null 041 cell extract was passed through: A) a column containing the SCS oligonucleotide, or B) a column containing the Sp1 binding site, and the presence of Sp1 in the elution fractions was detected by immunoblotting using Sp1 specific antibody PEP2. 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were loaded where indicated.
Nuclear extracts from the MDA 468 cells were passed through the column containing the p53 binding site found in the mdm2 gene. The presence of Sp1(C) or p53 (D) in the elution fractions was detected by immunoblotting. 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were loaded where indicated.

Transactivation of the HIV-LTR in MDA 468 cells is inhibited by the temperature sensitive p53 Val 135.

The tetrameric structure of p53 allows for the use of dominant negative alleles as a means to study its function. Assuming that mutant p53 His 273 contributes to the activation of HIV-LTR directed transcription as has been shown in co-tranfection experiments (Subler, 1994b) and figure 3.5A, we tested the ability of temperature sensitive (ts) p53 Val 135 to inhibit this activity of mutant p53 His 273 in MDA 468 cells. p53 Val 135 adapts a nuclear localized-wild type p53 conformation at 32°C but a cytoplasmic localized- mutant conformation at 37°C (Martinez, 1991). Since p53 forms tetramers, the association of the endogenous p53 with ts p53 Val135 at 37°C could prevent the nuclear importation of the endogenous p53. If the endogenous mutant p53 contributes to the transactivation of the HIV-LTR reporter, the decrease in endogenous p53 in the nucleus should lead to a decrease in the activity of the HIV-LTR reporter gene. Since wt p53 has been shown to repress HIV-LTR driven transcription, the activity of the HIV-LTR reporter should also decrease at 32°C. Stable cells lines were created by transfecting p53 Val135 into the MDA 468 cells, together with a plasmid expressing the neomycin resistant gene. Neomycin-resistant clones were selected and examined for the expression of p53 Val135 by Western blot analysis using p53-specific antibodies (Figure 3.5B). p53 Val135 can be differentiated from the endogenous p53 because it migrates faster on SDS polyacrylamide gels which might be due to polymorphic variability at codon 72 (Harris, 1986). Alternatively, the faster migration might be a property of mouse p53 -Val 135 is of mouse origin- but not of human p53. The activity of the HIV-LTR reporter gene was compared in cells stably expressing p53 Val 135 (cloned 8-9) to

that of the parental cell line. Figure 3.5C shows that at both 37°C and 32°C, the activity of the HIV-LTR reporter is significantly lower in the 8-9 clone which expressed p53 Val 135 than in the parental cells.





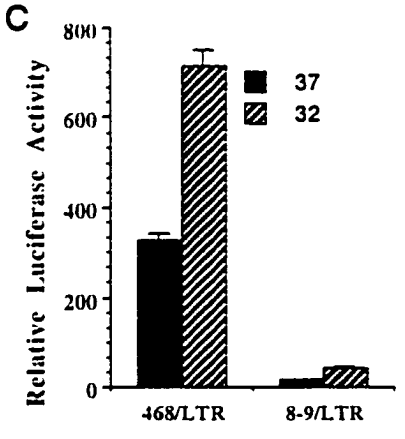


Figure 3.5. Transactivation of the HIV-LTR in MDA 468 cells is inhibited by the temperature sensitive p53 Val 135. **A)** Effect of mutant p53 in HIV-LTR-directed transcription in p53 negative 041 cells. **B)** Western blot of lysates from cells stably transfected with p53 Val 135. Lanes 1-4 contain lysates of neomycin resistant clones from ZR75.1 cells transfected with only the neomycin resistant gene (1-2) or co-transfected with Val 135 (2-4). Lanes 5-9 contain lysates of neomycin resistant clones from MDA 468 cells transfected with only the neomycin resistant gene (5-6) or co-transfected with Val 135 (7-9). Lysate in lane 10 is from p53 negative mouse fibroblast cells stably transfected with temperature sensitive p53 Val 135. **C)** Effect of stably transfected p53 Val135 on the activity of the HIV-LTR reporter. Parental MDA 468 cells and cells stably expressing the tsp53 Val135 (cloned. 8-9) were transfected with the reporter genes and treated as in 5A.

DISCUSSION

Association of mutant p53 with Sp1 binding sites occurs in cells endogenously expressing mutant p53 His 273 in the absence of growth factor stimulation.

Gualberto et al., identified an element in the HIV-LTR that is bound by p53 when Jurkat cells are treated with TNF-alpha (Gualberto, 1995a). They also showed that this cytokine induces Jurkat cells' p53 into a mutant-like conformation and that mutation of this element, which overlaps the Sp1 binding sites, inhibits mutant p53 transactivation of the HIV-LTR and TNF Alpha induction of HIV-LTR-directed transcription (Gualberto, 1995a). In contrast, tumor-derived mutants of p53 activate HIV-LTR-directed transcription (Subler, 1994b) and promote HIV replication in latently infected cells (Duan, 1994) in the absence of extra mitogen stimulation. Furthermore, mutant p53 His 273 and p53 His 175 purified from insect cells do not bind to the above DNA element as examined by EMSA (Bargonetti, 1997) suggesting that mutant p53 does not bind directly to this DNA element. Although it is not clear how mutant p53 activates transcription from the HIV-LTR, it is known that the Sp1 binding site region of the LTR is involved (Gualberto, 1995a)(Subler, 1994b). It would be expected therefore that under the circumstances where mutant p53 is activating transcription, Sp1 is contributing to the phenomenon through a DNA-associated multi-protein complex. We have shown that mutant p53 His 273 from the breast cancer cell line MDA-MB-468 associates with Sp1 when either Sp1 is bound to GC boxes or when p53 His 273 is bound to SCS DNA. We found that Sp1 did not bind to non-GC box DNA columns in the absence of p53 His 273 DNA binding activity. The ability of the two different types of DNA affinity columns to

isolate p53 His 273 and Sp1 suggests that this complex can be found on DNA when either of the two proteins is interacting with a specific sequence. Our data suggest that a DNA directed multi-protein complex exists at Sp1 binding sites consisting of Sp1, p53 and associated proteins. A number of different oncogenic mutant p53 proteins are able to activate the expression of a number of genes in addition to the HIV-LTR (Roemer, 1999). Although in this study we have not examined the interaction of other mutant p53 proteins with Sp1, our data suggest that this DNA associated complex does not occur between wild-type p53 and Sp1 as over-expressed wild-type p53 did not co-elute with Sp1 from the HIV-LTR column (Fig. 3. 2).

Wild type p53 and HIV-LTR-directed transcription

The observation that wt p53 did not co-elute with Sp1 from the DNA column indicates that wt p53, unlike mutant p53 His 273, does not associate with DNA bound Sp1. It has been shown, however, by DNAse footprinting and gel shift assays that wt p53 binds to the Sp1 binding sites of the HIV-LTR. In fact, wt p53 was shown to alter the binding of Sp1 and this was proposed as the mechanism for how wt p53 represses transcription from the HIV-LTR (Bargonetti, 1997). It is therefore intriguing that no p53 eluted from the Sp1 DNA column when extracts of cells expressing wt p53 were passed through this column. Two explanation can be given for these conflicting results. One invokes the nature of the assays and the other the source of p53. To detect the binding of a protein to DNA by DNA affinity, the protein must bind to DNA with high affinity (low dissociation constant). A protein with a high dissociation rate would be washed off the

column during the extensive washing carried out before the bound protein is eluted. Binding of a protein to DNA by gel shift or by footprinting can be detected even if the binding is transient (high dissociation constant) for binding is only being monitor at one specific time point. In gel shift, the protein-DNA complex becomes trap, in a process called "caging", when the complex enters the gel. During footprinting, the binding of the protein to DNA is only monitored at the moment when the DNAse is added. Alternatively, the difference can be due to the different sources of p53. The p53 used in footprinting and gel shift assays was purified from insect cells whereas the p53 used for DNA affinity was obtained form a human cell line.

Dominant negative effect of tsp53 Val 135 on the transactivation activity of oncogenic mutant p53.

Mutant p53 has been documented to inhibit the function of wild type p53 (Harvey, 1995; Kern, 1992; Zhang, 1993b). p53 is a tetramer so it is easy to envision how the association of a wild type peptide with a nonfunctional peptide could alter the activity of the wild type protein (Herskowitz, 1987). This dominant negative effect of mutant p53 on the wild type protein is only observed when mutant p53 is expressed at levels higher than the wild type protein (Harvey, 1995; Zhang, 1993b). Mutant p53 His273 has been shown to activate transcription of the HIV-LTR (Subler, 1994b). We have tested the ability of tsp53 Val135 to inhibit this transactivation activity of mutant p53 His 273 in the MDA 468 cells. The data showed that tsp53 Val135 was able to block the transactivation

activity of the endogenous mutant p53 at both 32°C and 37°C. At 37°C, p53 Val 135 might inhibit the activity of the endogenous mutant p53 by binding and preventing its nuclear localization as it has been shown that p53 Val 135 localizes to the cytoplasm at 37°C (Martinez, 1991). Nuclear importation of ts p53 Val 135 through is association with the endogenous p53 is unlikely as the association of mutant p53 with wt p53 has been shown to drive wt p53 into a mutant conformation (Milner, 1991). The inhibition at 32°C is most likely due to a direct effect of wild type p53 as it has been shown that wt p53 inhibits HIV-LTR directed transcription (Bargonetti, 1997). It could also be expected that the endogenous mutant p53 would inhibit the activity of the wild type protein; however, mutant p53 inhibits wild type p53 only when it is expressed at higher levels than the wild type protein (Harvey, 1995; Zhang, 1993b). As shown in figure 3.5B lane 5, this is not the case as p53 Val 135 is expressed at higher levels than the endogenous mutant p53 protein.

Model for mutant p53 transactivation.

Two models have been proposed to explain the ability of mutant p53 to activate transcription from the HIV-LTR. Gualberto et al. proposed that mutant p53 can activate transcription from the HIV-LTR by directly binding to DNA near the Sp1 binding sites (Gualberto, 1995a). Neither mutant p53 His 273 nor His 175 purified from insect cells bind to the Sp1 binding sites of the HIV-LTR as detected by EMSA (Bargonetti, 1997). Subler et al. have proposed an alternate model that suggests that mutant p53 activates transcription from the HIV-LTR by serving as a bridge between Sp1 and the general

transcription machinery (Subler, 1994). Our data is consistent with this model. A number of other studies validate the model in which non-DNA transcription factors can activate transcription of Sp1 containing promoters by associating with DNA-bound Sp1. The SMADS, which are intracellular signaling components of TGF-alpha, mediate TGF-alpha induction of the p21 gene by associating with DNA-bound Sp1 (Moustakas, 1998). c-Jun and the related family members JunB, and JunD also activate transcription from the p21(WAF1/CIP1) gene by associating with DNA-bound Sp1 (Kardassi, 1999). A role for p53 in activating transcription in the absence of DNA binding has also been demonstrated. Wild-type p53 can activate transcription of the GADD45 gene in the absence of direct DNA binding by forming a complex with WT1 (Zhan, 1998). Wild type and some mutants of p53 have been shown to activate transcription of GAL4 responsive reporter genes when fused to a GAL DNA binding domain (Raycroft, 1991). Together these data suggest that the oncogenic mutant p53 proteins might be able to activate the transcription of genes containing Sp1 binding sites through protein-protein complexes. Since Sp1 binding sites are commonly found in housekeeping and growth promoter genes, the increase expression of these growth promoting genes could be the mechanism for the gain of function of mutant p53.

Chapter 4

EGF-induced HIV-LTR transcription does not correlate with an EGF-induced mutant p53-Sp1 association

INTRODUCTION

Mutant p53 activates transcription of HIV-LTR driven reporters and of latent genomic integrated HIV virus (Subler, 1994b; Duan, 1994). Because mutant p53 does not bind to the HIV-LTR, it has been postulated that mutant p53 activates transcription from the HIV-LTR by associating with the transcription factor Sp1. Consistent with this, the region of the HIV-LTR containing the Sp1 binding sites is the minimal promoter element required for mutant p53 transactivation (Subler, 1994b) and we have detected a mutant p53 Sp1 complex on such a DNA element (Chapter, 3). In addition, an association between wt p53 and Sp1 has been detected in Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) treated cells (Borellini, 1993). It has also been shown that TNF-alpha can induce the association of p53 and Sp1 in Jurkat cells and that this complex synergized in activating HIV-LTR directed transcription (Gualberto, 1995a).

Since the growth factors stated above can induce the association of p53 and Sp1 and HIV-LTR directed transcription, we examined the ability of the epidermal growth factor (EGF) to induce this association and to enhance transactivation of an HIV-LTR reporter. This growth factor stimulates cell growth by binding and activating its receptor EGFR; a transmembrane glycoprotein with an extracellular EGF binding domain and an intracellular tyrosine kinase domain. Activation of this receptor leads to a signal transduction cascade through intracellular second messengers that ultimately promote cell growth (Carpenter, 1990; Fantl, 1993). MDA-MB-468 cells over-express the EGF

receptor (Filmus et al.. 1985) and therefore we expected that this cell line would be highly reactive to EGF treatment.

Here we present strong preliminary data indicating that EGF induces a mutant p53-Sp1 complex in MDA 468 cells but that this nucleoplasmic complex does not participate in activating transcription of Sp1 containing promoters.

RESULTS

EGF augments the association between mutant p53 and Sp1

We used the co-immunoprecipitation technique to examine the association of mutant p53 and Sp1 in MDA 468 cells. MDA 468 cells were treated for the time indicated with (100ng/mL) EGF and nuclear extracts were subjected to immunoprecipitation with a Sp1 antibody. A very small amount of p53 co-immunoprecipitated with Sp1 in cells not treated with EGF (Fig. 4.1A, lane 1) but the amount of p53 that co-immunoprecipitated with Sp1 dramatically increased after 6 hours of EGF treatment (Fig. 4.1A, lane 3). The increase in co-immunoprecipitation was not due to an increase of Sp1 immunoprecipitated at this time point. Densitometric analysis revealed that although there was a 3 fold increase in Sp1 immunoprecipitated at the six hour time point as compared to untreated cells, the amount of p53 co-immunoprecipitated at this time point was greater than 400 fold. The nuclear extracts were also examined by Western blot and densitometric analysis for their p53 and Sp1 levels (Fig. 4.1B). The level of both Sp1 and p53 remained relatively unchanged during the period of treatment indicating that the association was not due to changes in protein level.

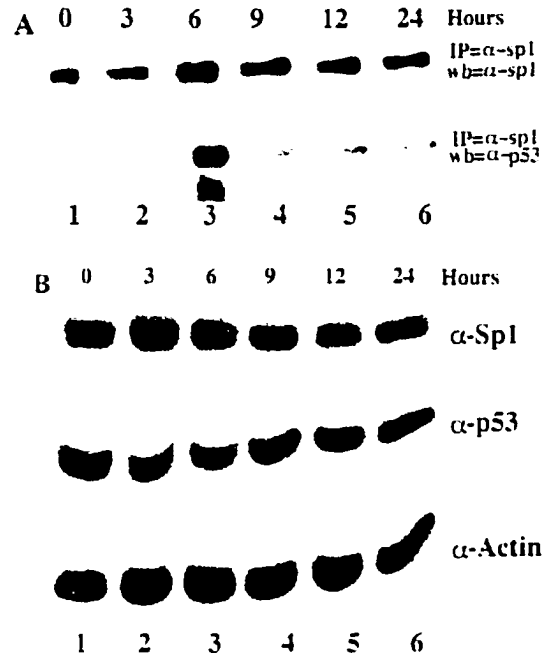


Figure 4.1. EGF induces an association of mutant p53 and Sp1 A). The MDA 468 cells were treated for the time indicated with recombinant EGF. Immunoprecipitations were carried out with the Sp1 polyclonal antibody PEP2G. The blot was probed with either the Sp1-specific antibody PEP2 or a mixture of p53-specific monoclonal antibodies. B) Western blot showing the levels of Sp1, p53 and Actin in nuclear extracts of cells treated with EGF. Although this curve was only done once, the six hour co-immunoprecipitation was repeated a least one more time as shown in figure 4.3.

EGF induces HIV-LTR directed transcription

To examine if the increased association between p53 and Sp1 could result in an increase in HIV-LTR-directed transcription. MDA-MB-468 cells were transfected with a luciferase reporter driven by the HIV-LTR and the transfected cells were treated with EGF for the indicated times (Fig. 4.2). The luciferase activity from cell extracts of EGF

treated cells was compared with the activity from extracts of untreated cells. A gradual increase in luciferase activity was detected as the time of EGF treatment increased but this increase activity did not correlate with the peak in co-immunoprecipitation which was observed after 6 hours of EGF treatment.

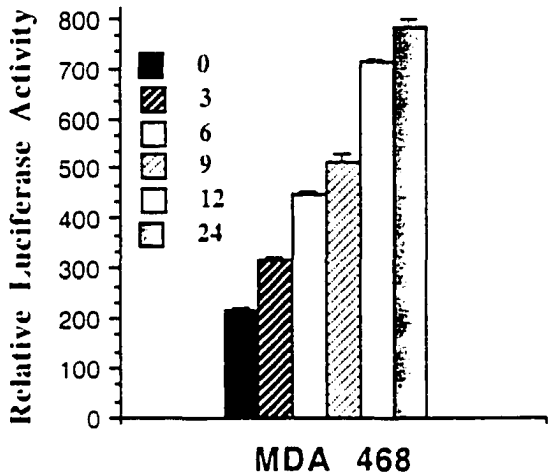


Figure 4.2. **EGF induces transcription of a HIV-LTR-driven luciferase gene.** MDA 468 cells were co- transfected with a luciferase reporter driven by the HIV-LTR and a plasmid expressing β-galactosidase. The activity of the luciferase reporter was examined 48 hours post-transfection and normalized for β-galactosidase activity. Where indicated, the transfected cells were treated with EGF (100ng/mL) for the time indicated. Error bars represent the standard deviation of triplicates. The experiment was carried out one time in triplicates

PKC inhibitors blocked the EGF-induced mutant p53-Sp1 association and EGF-induced transactivation of the HIV-LTR .

Protein kinase C (PKC) is an important mediator of EGF signaling as this kinase is activated by second messengers produced by the activation of the EGF receptor (Fantl, 1993). We therefore examined the role of PKC on the EGF-induced association of p53 and Sp1 and on the EGF-induced transactivation of the HIV-LTR. Immunoprecipitation with an antibody against Sp1 were carried out in extracts of cells treated with EGF for 6 hours in the presence or absence of PKC inhibitors. The co-immunoprecipitation of p53 with Sp1 was examined by Western blot analysis (Fig. 4.3A). p53 did not co-immunoprecipitate with Sp1 from extracts of cells not treated with EGF (4.3A, lane 2) but it co-immunoprecipitated from extracts of cells treated with EGF for 6 hours (4.3A, lane 3). Both the general PKC inhibitor GF10920X and the PKC-delta specific Rottlerin blocked the EGF-induced association of p53 with Sp1 as p53 did not co-immunoprecipitate with Sp1 in extracts of cells treated with these inhibitors (Figure 4.3A, lanes 4 and 5).

We also examined the role of PKC on the EGF-induced transactivation of the HIV-LTR. Transient transfections were carried out into the MDA 468 cells with the HIV-LTR luciferase reporter and the activity of the luciferase reporter was compared in extracts of cells treated with EGF for 6 hours in the presence or absence of the PKC inhibitors. As shown in Fig. 4.3B, both PKC inhibitors blocked the EGF-induced transactivation of the luciferase reporter. As a control experiment, we also examined the

effect of PKC inhibitors on the activity of the luciferase reporter in cells not treated with EGF. Both PKC inhibitors also decreased the activity of the luciferase reporter in the absence of EGF treatment.

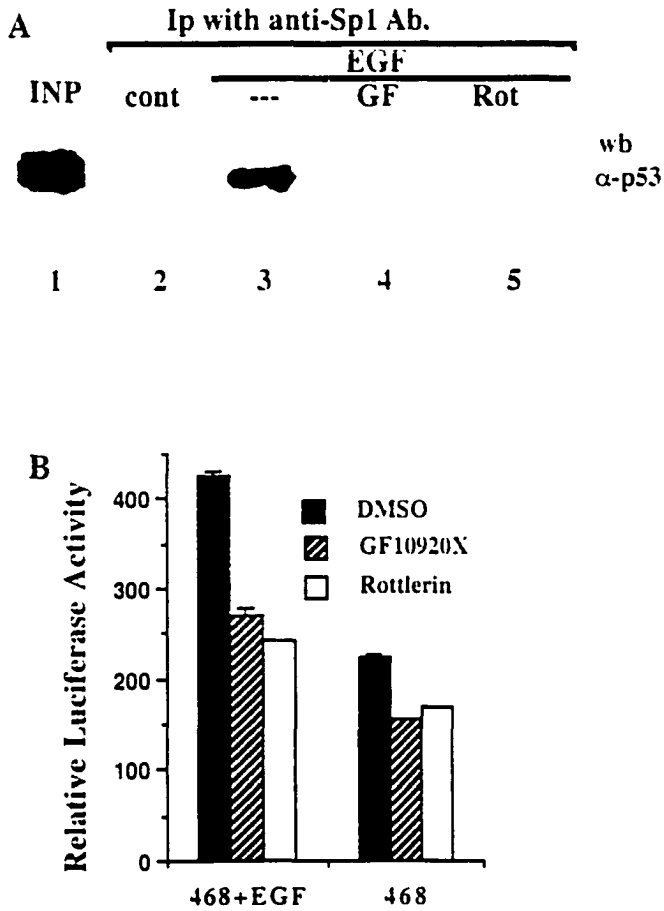


Figure 4.3. PKC inhibitors blocked the EGF-induced association of mutant p53 and Sp1. A) Extracts of cells not treated (lane 2) or treated with EGF alone (lane 3) or in the presence of either GF1092X (lanes 4) or Rottlering (lane 5) were subjected to immunoprecipitation with the Sp1 antibody PEP2G and the precipitates subjected to western blotting with a mixture of p53 antibodies. Lane 1 is 10% of the input. The experiment was performed once.

B) **PKC inhibitors blocked the EGF-induce transactivation of the HIV-LTR.** MDA 468 Cells were transfected with the HIV-LTR reporter and treated for six hours with EGF (100ng/mL) in the presence or absence of either 5 uM GF1092X or 1uM Rottlering. Cells not treated with EFG were also treated with the PKC inhibitors. Error bars represent the standard deviation of triplicates. The experiment was carried out one time in triplicates.

PKC inhibitors do not affect mutant p53 transactivation of the HIV-LTR in MDAH041 cells

The effect of PKC inhibitors on mutant p53 transactivation of the HIV-LTR in p53 minus cells was also examined. MDAH041 cells were transiently transfected with the HIV-LTR reporter alone or co-transfected with a plasmid expressing mutant p53 His 273 and the reporter gene. Mutant p53 increased the activity of the HIV-LTR luciferase reporter (Fig. 4.4) consistent with what was detected in another p53 negative cell line (Subler, 1994b). If PKC inhibitors were blocking a PKC-induced association of mutant p53 and Sp1 which was required for mutant p53 transactivation of the HIV-LTR, PKC inhibitors should block mutant p53 transactivation of the HIV-LTR in the 041 cells. The PKC inhibitors had no effect on the activity of the luciferase reporter transfected alone or co-transfected with mutant p53. These results indicate that in 041 cells PKC is not involved in mutant p53 transactivation of the HIV-LTR.

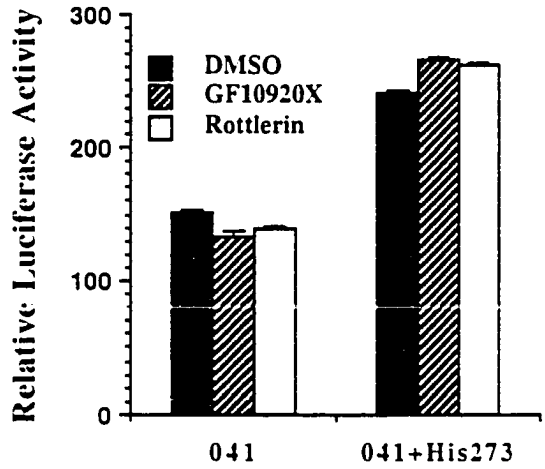


Figure 4.4. Effects of PKC inhibitors in the activity of the HIV-LTR/luciferase reporter in 041 cells. Cells were transfected with either the HIV-LTR luciferase reporter alone or with the reporter and the a mutant p53 His 273 expressing plasmid. Cells were treated with the PKC inhibitors for six hours before harvesting. Error bars represent the standard deviation of a triplicated experiment.

EGF induces an increase in p21 protein level that is independent of PKC

MDA 468 cells over-express the EGF receptor but treatment with EGF induces cell cycle arrest of these cells rather than proliferation (Filmus, 1985). EGF treatment also induces growth arrest of another cell line that over-expresses the EGF receptor and mutant p53 His 273 (Fan, 1995), namely the squamous breast carcinoma A431 line. In this cell line, the p21 cyclin kinase inhibitor has been shown to mediate the EGF-induced growth arrest (Ohtsubo, 1998). It was also shown by using specific inhibitors that this effect of EGF was independent of the MAP kinase pathway but dependent on the activity of PKC delta (Toyoda, 1998). Although p21 transcription can be activated in p53-

dependent and independent manners, it has been assumed that the induction of p21 in these cells is p53 independent because the p53 in these cells is mutant. Interestingly, the p21 gene has four Sp1 binding sites. We therefore examined whether the Sp1-mutant p53 complex could activate transcription of the p21 gene.

First, we determined whether the EGF-induced growth arrest in MDA 468 cells, like that of A431 cells, correlated with induced expression of p21. We showed that (100ng/mL) EGF induced a transient increase in p21 protein in the MDA 468 cells. The increase was first detected after 6 hours of EGF treatment, peaked between 9 and 12 hours and decreased to an undetectable level after 24 hours (Figure 4.5A). Interestingly, the six hour point correlated with the point at which the association between mutant p53 and Sp1 was detected. Since PKC inhibitors blocked the mutant p53-Sp1 association, we used PKC inhibitors to examine the role of PKC on the EGF-induced increase in p21 level. In contrast to what was shown in A431 cells, neither the general PKC inhibitor GF10920X (GF) nor the PKC delta specific inhibitor Rottlerin (Rott) were able to block the EGF-induced increase in p21 protein (Figure 4.5B). A slight decrease was however visually detected in the presence of Rottlerin (Rott) (Figure, 4.5B). Since PKC inhibitors blocked the EGF-induced mutant p53-Sp1 association without affecting the increase in p21 level, it is unlikely that the mutant p53-Sp1 complex is involved in the transactivation of the p21 gene.

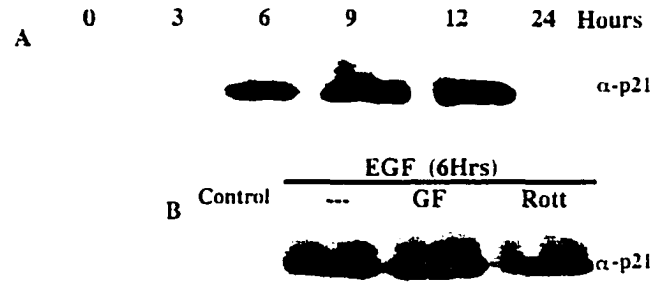


Figure 4.5. EGF induces an increase in p21 protein level that is independent of PKC A). MDA 468 cells were treated for the time indicated with recombinant EGF. Nuclear extracts from cells treated as indicated were made and analyzed by western blot analysis using a p21 specific antibody. B) Western blot of extracts made from cells treated with EGF for 6 hours in the presence or absence of the indicated PKC inhibitor. GF stands for the general PKC inhibitor GF10920X and Rott stands for the PKC delta specific inhibitor Rottlerin. Experiment was carried out once.

EGF induces transcription of a reporter gene driven by a p53 responsive element

We have also considered a direct role for p53 in the EGF-induced increase in p21 level based on the concept of re-activation. A number of studies have shown that some but not all mutants of p53 are conformationally flexible. Some mutants of p53 have been shown to be re-activated for sequence-specific DNA binding by factors such as temperature (Zhang, 1994; Friedlander, 1996), modifications of the carboxyl terminal regulatory domain such as deletion or binding of antibodies/synthetic peptides (Hupp, 1993; Selivanova, 1997), and by introducing mutations that created new contacts with the DNA (Wieczorek, 1996). Could an EGF-induced modification of p53 re-activate its transactivation activity? We have preliminary data suggesting that this is possible. We have transfected a luciferase reporter gene driven by the p53 responsive element in the

mdm2 gene into the MDA 468 cells and compared the activity of the reporter gene in cells treated or not treated with EGF. The activity of the reporter gene increased by 5 Fold in the presence of EGF (Figure 4.6). This reporter contains a TATA box in addition to the p53 responsive element and this effect of EGF could be mediated by the TATA box. This hypothesis can be tested by examining the activity of a reporter gene containing only the TATA box. This experiment and those proposed below (discussion) must be done to demonstrate that EGF can re-activate the DNA binding and transactivation activity of mutant p53 in MDA 468 cells.

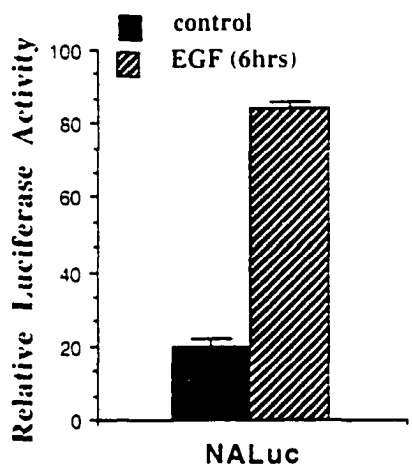


Figure 4.6. EGF induces transcription of a luciferase gene driven by the p53 responsive element from the mdm2 gene. MDA 468 cells were co- transfected with a luciferase reporter driven by the p53 responsive element from the mdm2 gene and a plasmid expressing β-galactosidase. The activity of the luciferase reporter was examined 48 hours post-transfection and normalized for β-galactosidase activity. Where indicated, the transfected cells were treated with EGF (100ng/mL) for 6 hours. Error bars represent the standard deviation of triplicates. The experiment was carried out one time in triplicates.

DISCUSSION

EGF induces a mutant p53-Sp1 complex that does not activate transcription of Sp1 containing promoters

An association between His 273 p53 and Sp1 was detected by DNA affinity chromatography (Chapter 3). This association, however, was barely detected when nuclear extract from normally cultured cells was examined by co-immunoprecipitation (Figure 4.1). This suggested that only a small fraction of these proteins were capable of associating and/or that the association might only take place in DNA. There is ample precedent for the requirement of DNA in the formation of a multi-protein complex (Klein, 2000; Chandran, 1999; Boehmer, 1998; Ting, 1999; Gomes, 2000; Basuyaux, 1997; Gonzalez, 2000). An interesting and complex example is that of the role of Oct-1 in gene regulation by steroid hormone receptors. Steroid receptors such as androgen (AR) and glucocorticoid (GR) recognize similar DNA binding sites but their activation elicit distinct responses (Gonzalez, 2000). Both AR and GR can bind *in vitro* to a hormone response element (HRS) in the sex-limited protein (slp) gene but only AR activates its transcription *in vivo*.. This is due to the fact that only AR can associate with Oct-2 bound to a Oct-2 site located adjacent to the HRS. It is interesting to note that although both AR and GR can co-immunoprecipitate with Oct-2 only AR can associate with DNA-bound Oct-2 (Gonzalez, 2000). Another interesting example is the "nucleation" of multi-protein complexes by DNA elements such promoters, enhancers and silencers.

Although the limited interaction detected by DNA affinity chromatography might be what accounts for the activation of HIV-LTR transcription in MDA 468 cells (Chapter

3), an increased association should be able to increase the level of activation. Since factors such as TNF-alpha can induce an association of p53 and Sp1 that can be detected by co-immunoprecipitation (Gualberto, 1995a), we examined the ability of EGF to induce the association of mutant p53 and Sp1 in MDA 468 cells. We have shown that EGF is able to mediate the formation of a His273 p53-Sp1 complex. We found that not only did EGF mediate an increase in the association of His273 p53 with Sp1 but also caused activation of HIV-LTR driven transcription. The correlation, however is less than perfect. Although EGF induces the maximum Sp1-p53 association after six hours of EGF treatment, there is no peak reporter gene activity even after 24 hours of EGF treatment. This is in contrast to what was reported in Jurkat cells in which the TNF alpha induced association of mutant p53 and Sp1 was correlated with increase HIV-LTR driven transcription (Gualberto, 1995a, 1995b). Close examination of the latter data, however, reveals that the immunoprecipitation were carried out after 1 hour TNF alpha treatment whereas the reporter gene assays were carried out after 16 hours of treatment (Gualberto, 199a, 1995b). It is possible therefore that the nucleoplasmic-growth-factor induced p53-Sp1 complex that is detected by immunoprecipitation does not activate transcription from the HIV-LTR; possibly because this nucleoplasmic-complex does not bind to DNA.

Other transcription factors such as the SMADS, JunB and JunD have been shown to activate transcription of genes containing Sp1 binding sites by associating with Sp1 (Moustakas, 1998; Kardassi et al., 1999). The EGF-induced mutant p53-Sp1 complex could, therefore, be involved in activating transcription of genes containing Sp1 binding sites other than the HIV-LTR. We examined whether the mutant p53-Sp1 complex could

activate transcription of the p21 gene; a gene whose promoter has five Sp1 binding sites. Although a EGF-induced increase in p21 levels was detected, this did not correlate with the formation of the mutant p53-Sp1 complex induced by EGF. PKC inhibitors blocked the EGF induced mutant p53-Sp1 association but not the EGF-induced increase in p21 protein level. Interestingly, the PKC inhibitors blocked the EGF-induced HIV-LTR directed transcription at the six hour time point. This suggested that the complex might be involved in activating transcription at this time point. However, the PKC inhibitors also blocked the HIV-LTR transcription in the absence of EGF treatment. It must be considered, however, that there is some mutant p53-Sp1 association in the absence of EGF treatment as detected by DNA affinity chromatography. The PKC inhibitors could be blocking this "basal" association and hence the reason for the reduction in the HIV-LTR reporter activity. If this was the case, PKC inhibitors would be expected to inhibit mutant p53 transactivation in 041 cells. Contrary to this, in the p53 negative 041 cells, PKC inhibitors showed no effect on the ability of mutant p53 His 273 to activate transcription from the HIV-LTR.

Although a number of cytokines have been shown to induce HIV-LTR-directed transcription (Vicenzi et al., 1997), this is the first time that EGF was shown to enhance HIV-LTR-directed transcription. Even if mutant p53 is not involved in the EGF-induced activation of transcription, it is clear from our data that this effect of EGF is PKC dependent. It would of be interest to determine which PKC isoform mediates this effect and whether the MAP kinase pathway is involved. Also, the identification of the EGF

responsive element in the HIV-LTR is important to understand the mechanism of EGF-induced transactivation.

Re-activation of mutant p53

Besides gene therapy, re-activation of the wild type function of mutant p53 has been the most attractive anti-cancer therapeutic strategy. Originally, this was thought to be an impossible task due to the nature of the alterations in p53 (Friend, 1994). Mutations in p53 are targeted to amino acid residues that either make contact with DNA or amino acid residues that allow for the proper folding of the protein (Cho, 1994). Re-activation would require making new contacts with the DNA or refolding a denatured protein. A number of studies have shown, however, that some but not all mutants of p53 are conformationally flexible. Some mutants of p53 have been shown to be re-activated for sequence-specific DNA binding by factors such as temperature (Zhang, 1994; Friedlander, 1996), modifications of the carboxyl terminal regulatory domain such as deletion or binding of antibodies/synthetic peptides (Hupp, 1993; Selivanova, 1997), and by introducing mutations that created new contacts with the DNA (Wieczorek, 1996). Similar to mutant p53, deletion of the carboxyl terminal regulatory domain or binding of antibodies/synthetic peptides to the carboxyl terminus can also activate the latent sequence-specific DNA binding activity of wild type p53. In contrast, wild type but not mutant p53 can be activated for sequence-specific DNA binding by phosphorylation of the carboxyl terminus of p53 (Hupp, 1994).

We have begun a study to examine re-activation of mutant p53 and have preliminary evidence showing that EGF can re-activate the sequence-specific

transactivation activity of mutant p53 His 273 (Figure 4.6). This mutant p53 has been shown to be re-activated by deletion of the carboxyl terminal region as well as by the binding of antibody 421 to this region (Hupp, 1993; Wieczorek, 1996). Further analysis will reveal whether this is truly the case.

Questions to be answered?

1). What is the minimal DNA element required for the EGF-induced increase in p21?
Strategy:
Comparing the activity of luciferase reporters driven by progressively smaller regions of the p21 gene in EGF treated 468 cells.

2). Is the increase in the reporter gene activity due to the binding of p53?
Strategy:
DNA binding assays can be used to show that EGF treatment re-activated the DNA binding activity of mutant p53 His 273.

3). Is the transcription of other p53 responsive genes also activated by EGF?
Strategy:
Northern blot analysis of RNA obtained from EGF treated 468 cells using probes specific for the different p53 responsive genes.

Chapter 5

Preliminary Data for remaining questions

Section I
DNA binding activity of p53 expressed from an inducible promoter

Introduction

p53 is a sequence-specific transactivator that modulates the cellular stress response and under non-stress conditions is not detected by common detection techniques. Two experimental systems are commonly used to increase the level of p53 in order to study its function. One is the stabilization of p53 by DNA damage and the other is the over-expression of p53 from an inducible promoter. It is well documented that the activation of the latent sequence specific DNA binding activity of p53 requires modifications of p53 at the regulatory carboxyl terminal domain (Giacca, 1997). These modifications are also induced simultaneously by the same DNA damage that causes stabilization of p53 (Chapter 1). These modifications would not be expected to be made in p53 when it is expressed from an inducible promoter. Yet, over-expression of p53 from an inducible promoter is sufficient to detect sequence specific DNA binding of p53 in gel mobility shift assays and to detect transactivation of some but not all wild type p53 responsive genes (Xiao, 2000). This contradicts the well established concept that sequence-specific DNA binding of p53 requires modifications of the carboxyl terminus.

Results

We have compared the DNA binding activity of p53 in the presence and absence of DNA damage by gel mobility shift assay and DNA affinity chromatography. We used the TR9-7 cell line which expresses p53 from a tetracycline regulated promoter. p53 is induced by removing tetracycline for 24 hours. The DNA damage signal is initiated by

treating the cells with 100uM camptothecin for 4 hours before harvesting. Figure 5.1A shows that p53 from cells treated or not treated with camptothecin binds equally to the SCS. The binding is specific as it is only competed with excess cold SCS oligonucleotide but not by cold excess mutant (mt) oligonucleotide. The binding of p53 (complex labeled p53) is super-shifted by the p53 specific antibody 1801 (complex labeled Ab/p53) but not the p53 specific antibody 421. This is not surprising since p53 from these cells is not reactive to 421 as seen by western blot (data not shown). Interestingly, the 421 antibody is routinely used to activate the latent DNA binding activity of p53. It has been shown *in vitro* that PKC phosphorylation of p53 activates the DNA binding activity of p53: an event that blocks recognition of p53 by the 421 antibody because the 421 epitope overlaps the PKC phosphorylation site (Takenaka, 1995). This suggested that the p53 in TR9-7 cells was PKC phosphorylated. Treatment of these cells with PKC inhibitors, however, did not restore the 421 reactivity of p53 (data not shown).

To examine the DNA binding activity of p53 by DNA affinity chromatography, TR9-7 nuclear extracts from cells grown in the absence of tetracycline and either treated or not treated with camptothecin were passed through a SCS/DNA column. The presence of p53 in the elution fractions was tested by gel mobility shift assay. When nuclear extract of cells treated with camptothecin was passed through the SCS DNA column, p53 was detected in the 0.3 and 0.4M KCl elution fractions (Figure 5.1B) indicating that p53 bound to the DNA column. When nuclear extract of untreated cells was passed through the column, p53 was not detected in any of the elution fractions (Figure 5.1C) indicating that p53 did not bind to the DNA column. This is in contrast to the results obtained by

gel mobility shift assays in which p53 from both treated or untreated cells bound equally to DNA.

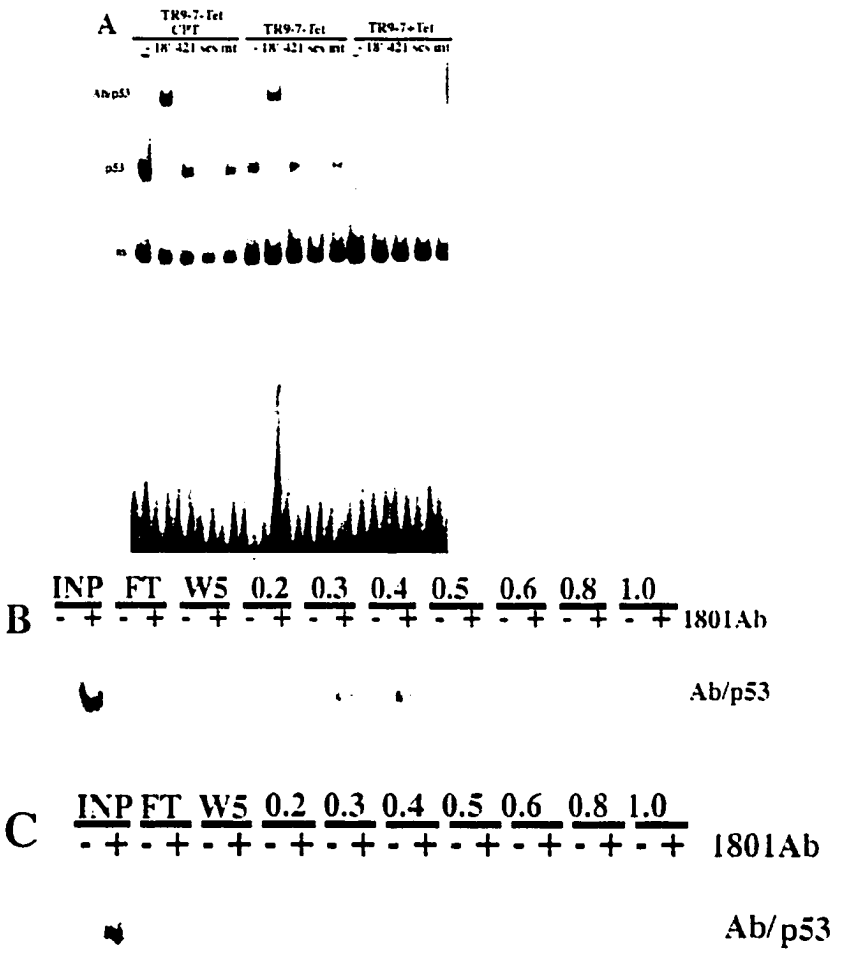


Figure 5.1. DNA damage is required for activation of wt p53 DNA binding in a DNA affinity chromatography assay but not in a gel mobility shift assay. A). EMSA showing the binding of wt p53 in TR9-7 cells to the super consensus sequence (SCS) oligonucleotide. Binding is super-shifted by the p53 specific antibody 1801 (18') but not by antibody 421. Binding is competed with excess unlabeled SCS oligonucleotide but not by excess mutant oligonucleotide (Mut). B&C) Nuclear extracts of TR9-7 cells grown in the absence of tetracycline and treated (B) or not treated (C) with camptothecin (CTP) were passed through the SCS DNA column and the presence of p53 in the elution fractions tested by EMSA. Only the super-shifted species is shown (Ab/p53).

DISCUSSION

The inability of p53 derived from cells not treated with camptothecin to bind to DNA in the DNA affinity column assay is an intriguing result. Since this p53 binds to the same DNA element in gel shift assays, this result suggests that the gel shift conditions activate the sequence specific DNA binding activity of p53. The phenomenon of "caging", which is a characteristic of gel shift assay but not DNA affinity chromatography, might also contribute to this difference. Before beginning to look for explanation however, the experiment must be repeated to be certain of its reproducibility. If this result is reproducible, it could lead to a number of interesting questions. It has been recently proposed that the function of p53 under non-stress conditions is to bind and repair damaged DNA (Janus, 1999). In contrast, the function of p53 under stress conditions is to bind sequence-specifically to DNA and activate transcription of its target genes. Our preliminary data showed that p53 derived from stress cells (therefore activated) can bind to the SCS DNA; that is , it can bind to DNA in a sequence-specific manner. Our preliminary data also showed that under DNA affinity assay conditions, the p53 derived from non-stress cells (dormant/inactive p53) did not bind to the SCS DNA. The model predicts that p53 derived from non-stress cells should be able to bind to damaged DNA. This can be tested by passing p53 from non-stress cells through a column containing damaged DNA; for example a column containing DNA mismatches. The model also predicts that p53 derived from stress cells is unable to bind to damaged DNA. This can also be tested by passing p53 derived from stress cells through a column containing damaged DNA.

Section II

DNA binding activity of mutant p53

Introduction

A gain of function activity of mutant is frequently described in the p53 literature (Roemer, 1999). One of the models of mutant p53 gain of function proposes that this activity is mediated by the ability of mutant p53 to bind to DNA elements not bound by wild type p53. This model is mainly derived from the observation that mutant p53 has a higher affinity for Matrix Attachment Regions (MAR) than wt p53 (Will, 1998). MAR DNA elements modulate important cellular processes such as gene expression, replication, and recombination (Review by Deppert, 1996). Binding of mutant p53 to these regions might, therefore, influence all of these processes

We have examined the DNA binding activity of mutant p53 to a number of DNA sequences by DNA affinity chromatography to test the specificity of the high affinity DNA binding activity of mutant p53. We have compared the binding activity of a type II conformational mutant (His 175) to a type I contact mutant (His 273). We have also compared the ability of mutant p53 expressed in either insect cells or human cells to bind to the different DNA elements.

Equal amounts of p53, expressed either in insect cells or in MDA 468 cells, were passed through DNA columns containing different DNA sequences and the presence of p53 in the elution fractions examined by Western blot analysis. The presence of p53 in the elution fractions is an indication that p53 bound to the DNA columns. Importantly, no p53 was detected in any of the elution fractions when a beads-only column was used

(data not shown). The conformational mutant p53 His 175 expressed in insect cells bound with equal affinity to a DNA affinity column containing the Sp1 binding sites of the HIV-LTR (5.2A) and to a DNA column containing a mutated p53 binding site (5.2B). Likewise, the contact mutant p53 His 273 expressed in insect cells bound to the LTR/DNA column (5.2C), the RGC mutant DNA column (5.2D), and the SCS/DNA column (5.2E). There was no significant difference in the binding to the different DNA element. There were, however, differences in the elution profiles for the two mutants (compare 5.2A to 5.2C). This might be a reflection of different affinities of the different mutants for DNA.

There were significant differences in the DNA binding activity of mutant p53 His 273 when the source of p53 was the human breast cancer cell line MDA 468. This mutant p53 bound well to the column containing the p53 super consensus sequence(5.2H) but not to the LTR/DNA column (5.2F) or a column containing the p53 responsive element of the mdm2 gene (5.2G). Specific binding of this mutant p53 to the p53 super consensus sequence has been documented by other laboratories (Zhang, 1993; Prasad, 1997). Some p53 was detected in the elution fractions of the LTR/DNA column when the membrane was over-exposed. We have shown that this is due to the ability of mutant p53 to associate with DNA bound Sp1 and not to direct binding of mutant p53 to the HIV-LTR.

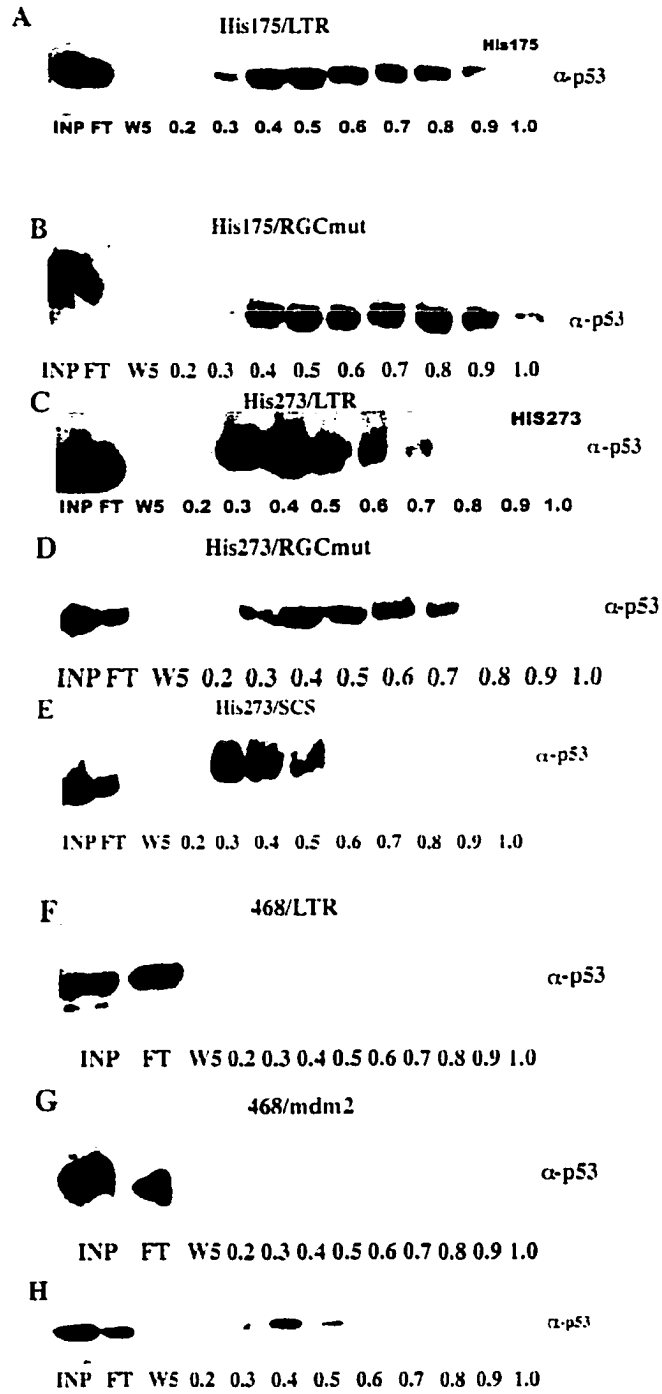


Figure 5.2. Mutant p53 expressed in Insect cells binds to DNA differently than mutant p53 in human tumor-derived cell lines. Equal amounts of mutant p53 expressed in either insect cells or from the MDA 468 cells was passed through the indicated DNA column and the elution fractions examined for the presence of p53 by

western blot analysis using a mixture of p53 monoclonal antibodies. A&B). Insect cells expressed His175 was passed through either the LTR DNA column (A) or MutRGC DNA column (B). C-E). Insect cells expressed His 273 was passed through either the LTR DNA column(C), the MutRGC DNA column (D), or the SCS DNA column (E). F&G). His 273 from the MDA 468 cells was passed through either the LTR DNA column (F), the mdm2 DNA column (G) or SCS DNA column (H).

Discussion

We have examined the DNA binding activity of mutant p53 by DNA affinity chromatography. The data indicate that mutant p53 expressed in insect cells has high DNA binding activity that is non-specific as the mutants did not discriminate among the different oligonucleotides. Both types of mutants, conformational and contact as represented by His 175 and 273 respectively, have this activity. It is intriguing that this non-specific DNA binding activity of mutant p53 is not detected in gel mobility shift assays as neither His 175 nor His273 bound LTR/DNA when examined by EMSA (data not shown). In contrast, sequence-specific DNA binding was detected by EMSA as binding of mutant p53 His 273 to the SCS oligonucleotide was detected by gel mobility shift assays (data not shown). These data prompted the question of whether wild-type p53 expressed in insect cells would also have high non-specific DNA binding activity. Preliminary data indicates that insect cells-expressed wild type p53 does not bind to a DNA column containing a mutated RGC site but that it bound to a column containing a wild type RGC site (data not shown). These data suggest that mutant p53 has higher non-specific DNA binding activity than wild type p53. Further experiments using columns

containing other sites must be carry out before a conclusion is made about these observations.

The high non-specific DNA binding activity of mutant p53 His 273 is inhibited when this is expressed in MDA 468 cells. Mutant p53 His 273 from MDA 468 cells only bound the column containing SCS DNA; a site to which mutant p53 His 273 binds in a sequence specific manner (Chapter 3). It would be of interest to determine how the non-specific DNA binding activity of mutant p53 His 273 is inhibited in MDA 468 cells and whether this inhibition is a common feature in mammalian cells. Is the inhibition due to a factor competing for binding or to modification(s) of p53? To determine if the inhibition of non-specific DNA binding activity is a common feature among mammalian cells, extracts from insect cells expressing mutant p53 can be mixed with extracts of mammalian cells not expressing p53 and the ability of mutant p53 to bind to DNA examined by DNA affinity chromatography. To determine if the inhibition of non-specific DNA binding activity in mammalian cells is due to a DNA competing factor, nuclear extract from mammalian cells can be passed through the column prior to passing the insect cells extract. If a DNA competing factor is present in the mammalian cell extract, it would bind and saturate the DNA sites in the column, therefore preventing the binding of mutant p53.

Section III

Wild type p53 repression of HIV-LTR-driven transcription

Introduction

A number of viral and cellular promoters have been shown to be repressed by the over expression of wild-type p53. Viral promoters that are repressed by p53 include SV40 (Jackson, 1993; Subler, 1992), herpes simplex virus thymidine kinase and UL9 promoters (Deb, 1992), human cytomegalovirus-immediate-early promoter (Deb, 1992) and the long terminal repeat regions (LTR) of HIV, Rous sarcoma virus, and human T-cell lymphotropic virus (Deb, 1992; Subler, 1992). Cellular promoters that have been shown to be repressed by wild-type p53 include the MDR1 promoter (Chin, 1992), the PCNA promoter (Deb, 1992; Mercer, 1991), the c-fos promoter (Ginsberg, 1991; Kley, 1992), c-myc promoter (Moberg, 1992) and gene (Ramigov; 1992), the promoters of interleukin 2, 4 and 6 (Pesch, 1996; Margulies, 1993), the mouse thymidylate synthase promoter (Lee, 1997), the Rb promoter (Shiio, 1992; Santhanam, 1991), and the basic fibroblast growth factor (bFGF) promoter (Ueba, 1994). Although, the above studies were carried out under conditions of artificial overexpression of both p53 and target promoters, it has also been shown that physiological induction of wild-type p53 repressed transcription of the gene encoding the microtubule-associated protein MAP4 (Murphy, 1996).

Although no p53 binding sites have been identified in the cellular genes repressed by p53, wild-type p53-mediated transcriptional repression is promoter specific as some promoters such as the MHC1promoter are not affected. (Ragimov, 1992). Some have suggested that the repression is specific for TATA box containing promoters (Mack, 1993) despite the fact that TATA-less promoters are also repressed by wt p53 (Iotsova, 1996). Sp1 binding sites are known to be present in a number of the promoters repressed

by wt p53 (Jackson, 1994; Moberg, 1992; Kley, 1992; Deb, 1992) and it has been proposed that p53 represses transcription by disrupting the binding of transcription factor Sp1 to DNA (Perrem, 1995; Bargonetti, 1997). However, not all promoters repressed by wt p53 contain Sp1 binding sites

Because repression of transcription by p53 is observed under conditions in which p53 is over-expressed, this repression could be the result of "squelching" by p53. Squelching is the titration of molecules involved in transcription by excess transactivator (Gill, 1988) thereby lowering the expression of other promoters not containing binding sites for that particular transactivator. Because p53 has been shown to interact with TBP (Martin, 1993; Truant, 1993; Liu, 1993), it has been suggested that wild type p53 represses transcription by sequestering TBP (Seto, 1992). This suggestion is inconsistent with the observation that the mutant p53 $_{gln22, ser23}$ which is unable to repress transcription of the c-fos or SV40 early promoters (Roemer, 1996) can still associate with TBP (Lin, 1994). Interestingly, this mutant p53 is unable to interact with the $TAF_{II}40$ and $TAF_{II}60$ components of the TFIID complex (Thut, 1995). These data suggest that sequestering these co-activators might be the mechanism by which wt p53 represses transcription. Consistent with this, it has been shown that supplementation of intact TFIID but not recombinant TBP was able to rescue self-squelching by p53 in an *in vitro* transcription assay (Liu, 1995).

It is also possible that the repression of transcription observed *in vivo* is the result of p53-induced cell cycle arrest or apoptosis. In fact, similar to p53, over-expression of the p53- inducible gene bax has been shown to repress transcription of the c-fos promoter (Ryan, 1998). No transcriptional function has been attributed to this cytoplasmic protein

known for inducing apoptosis (Antonsson, 1997). Consistent with this latter but not with squelching, some tumor-derived mutants of p53 that retain the ability to bind to the TFIID components but can not induce cell cycle arrest or apoptosis do not repress transcription (see for instance Deb, 1992; Subler, 1992). In fact, some of these mutants activate transcription of the same promoters that are repressed by wt p53 (Deb, 1992; Subler, 1992). In addition, most of the cellular promoters that are repressed are those involved in cell cycle progression (c-fos, myc, PCNA etc). This posses the question of whether repression of transcription is the consequence of cell cycle arrest or whether repression of transcription contributes to cell cycle arrest. Some of the repressed promoters, however, have also been shown to be repressed by p53 in transcription assays *in vitro* (Ragimov, 1992; Seto, 1992; Mack, 1993) indicating that this is a direct effect of p53 and not the consequence of p53-induced cell-cycle arrest or apoptosis.

We have used the HIV-LTR to examine p53-mediated repression of transcription. We consider the question of whether the repression is the result of the growth arrest induced by wt p53. We used a tetracycline-inducible cell line to examine p53 mediated repression without the need for co-transfecting wt p53 with the reporter plasmid.

Results

As stated above, over-expression of wt p53 has been shown to repress HIV-LTR directed transcription in the p53 negative cell line Soas2 (Subler, 1992). We have used a different p53 negative cell line, namely the MDH041 cells, to reproduce these results. The wt p53 expressing plasmid was co-transfected by electroporation into the 041 cells with a luciferase reporter driven by the HIV-LTR. As a control experiment, the

luciferase reporter was transfected alone. The luciferase activity was determined and compared in lysates of cells transfected with or without p53. As shown in Figure 5.3, the presence of p53 reduced the activity of the luciferase reporter by a factor of 5. These results agree with those published by Subler. It has been shown that over-expression of the p53 inducible gene bax repressed transcription of the fos promoter (Ryan, 1996). Since Bax protein does not possesses transcriptional function, this is most likely a consequence of the apoptotic process induced by Bax. Since p53 also induces apoptosis, p53-mediated repression may also be a consequence of apoptosis. In cells in which wt p53 does not induce apoptosis but, rather, induces growth arrest, repression of transcription by wt p53 could be due to this growth arrest. To examine the effect of cell cycle arrest on HIV-LTR directed transcription, we co-transfected the HIV-LTR/Luciferase reporter into 041 cells with a plasmid encoding the p21 protein. Figure 5.3 shows that unlike wt p53 that repressed transcription 5 fold, p21 over-expression did not repress transcription of the luciferase reporter but rather appeared to enhance it.

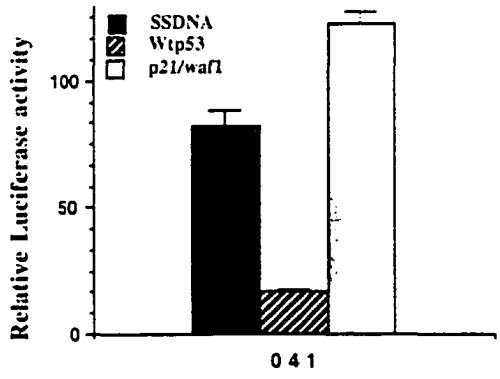


Figure 5.3. Wt p53 repression of HIV-LTR driven transcription is not a consequence of p21-induced cycle arrest. The HIV-LTR luciferase reporter was transfected alone or together with either a p21 expressing plasmid or a wt p53 expressing plasmid into the 041 cells. The amount of DNA was normalized with salmon sperm DNA (SSDNA). Although all the transfections included a b-galactosidase plasmid. normalization was to total protein concentration because wt p53 also repressed transcription of b-galactosidase. This experiment was carried out once and the expression of the p21 protein was not examined after transfection.

The effect of transfection on the ability of p53 to repress transcription was examined by using the tetracycline-regulated wild-type p53 expressing cell line TR9-7 (Agarwal, 1995). This cell line is an isogenic line derived from MDAH041 cells in which the expression of p53 is regulated by tetracycline. As shown in the inset of Figure 5.4, no p53 is detected in lysates of these cells grown in the presence of tetracycline but p53 is detected 24 hours after removal of antibiotic. TR9-7 cells were transfected by electroporation with the HIV-LTR/Luciferase reporter. The activity of the reporter was compared in lysates of transfected cells maintained in the presence of tetracycline with cells in which tetracycline was removed for 24 hours. Surprisingly, no significant difference was detected in the activity of the reporter in the presence or absence of p53. As expected, the activity of a luciferase reporter driven by the p53 responsive element found in the mdm2 gene increased in the presence of p53 (Figure 5.4).

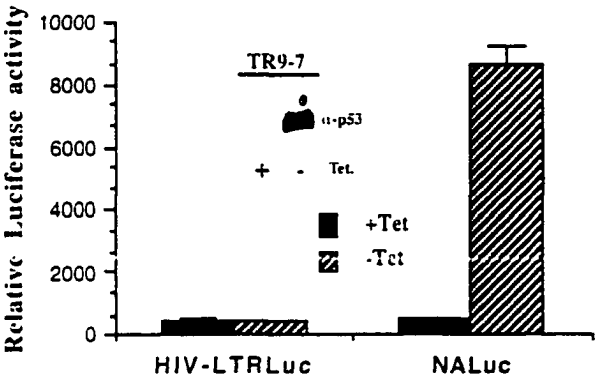


Figure 5.4. Wild type p53 expressed from an inducible promoter does not repress HIV-LTR driven transcription. TR9-7 cells were transfected with luciferase reporters driven by either the HIV-LTR or the p53 responsive element from the mdm2 gene. Where indicate, tetracycline (-Tet) was removed from the media 24 hours before harvesting the cells. Inset. Western blot showing the level of p53 in TR9-7 cells grown in the presence or absence of tetracycline.

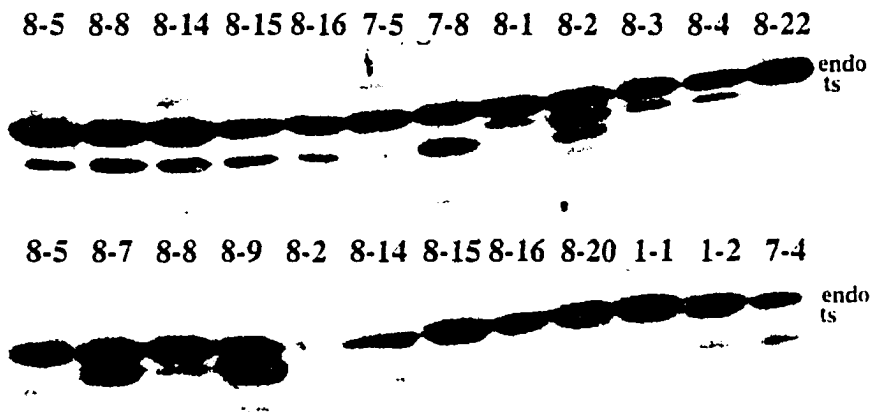
DISCUSSION

Over-expression of wt p53 has been shown to repress HIV-LTR-driven transcription in transient transfection assays (Deb, 1992; Subler, 1992). We have expanded those results and have shown that the repression is not due to the cell cycle arrest that is induced by over-expressed wild type p53. We also

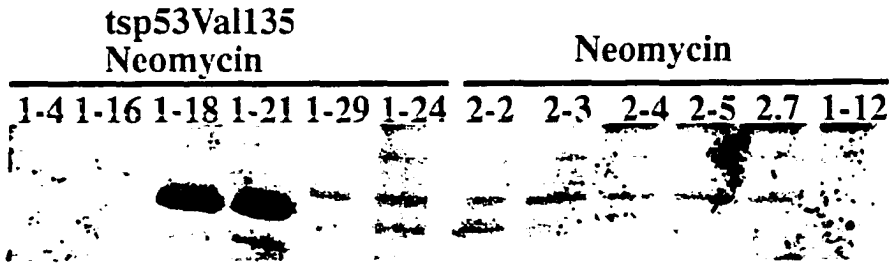
showed that in the TR9-7 cell line, which expresses high levels of p53 from an inducible promoter, the repression of HIV-LTR-driven transcription is not observed. This was unexpected because the parental cell line 041was capable of p53 mediated repression of the HIV-LTR in co-transfection experiments. One explanation for this difference could be that the amount of p53 transfected into the 041 cells was higher than the amount of p53 expressed from the TR9-7 cells. This possibility can be tested by determining the minimum amount of p53 that must be transfected into 041 cells to detect repression of HIV-LTR directed transcription and comparing it to the amount of p53 expressed in TR9-7 cells. If the amount of p53 that causes repression in 041 cells is less than the amount expressed in TR9-7 cells, it would indicate that p53 mediated repression is a consequence of the transfection of p53.

Chapter 6

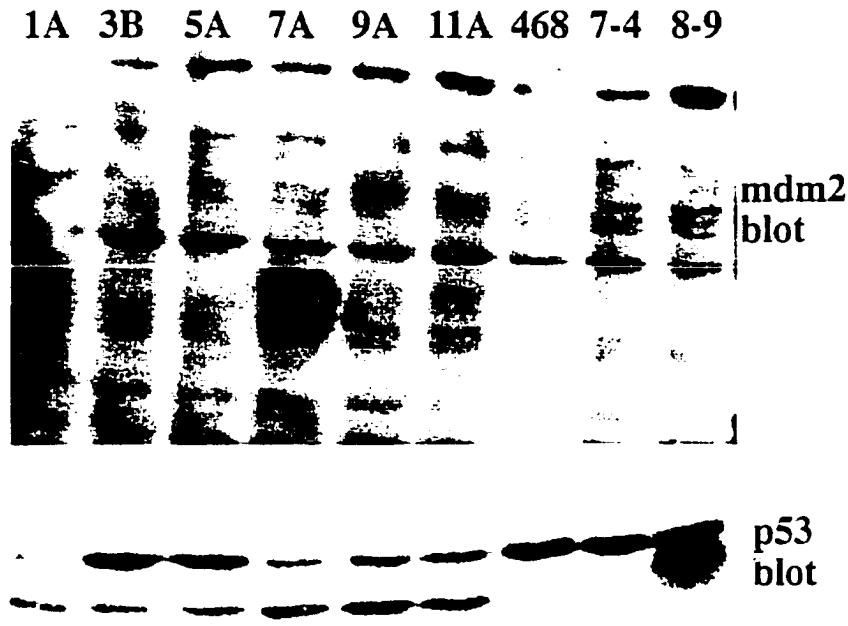
APPENDIX



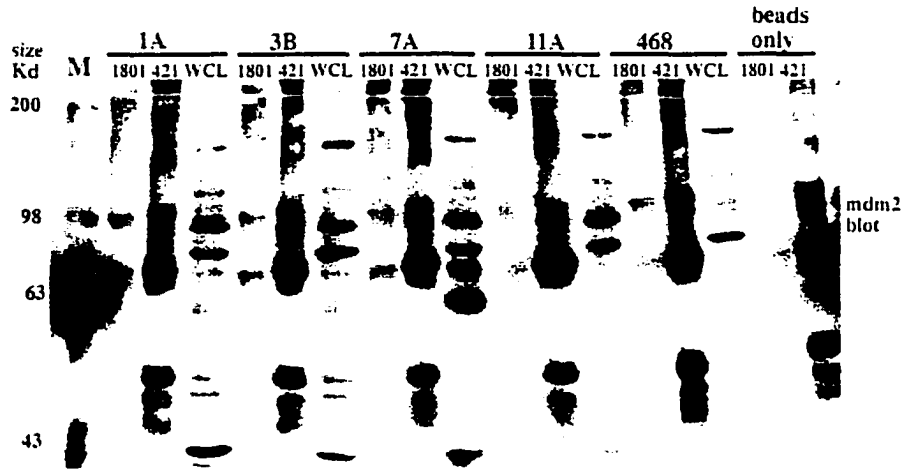
6.1 Clones from MDA 468 cells transfected with the murine temperature sensitive p53 Val135. Cells were transfected with a plasmid expressing p53 Val135 and a plasmid expressing the Neomycin resistance gene or with the Neomycin resistance gene alone. Neomycin resistant clones were selected in media containing G418 (0.2mg/mL). Expression of p53 Val135 was monitored by western blot analysis of cell lysates with a mixture of p53 monoclonal antibodies.
p53 Val 135 (ts) is distinguished from the endogenous p53 (endo) by its faster mobility on a 10% SDS polyacrylamide gel.



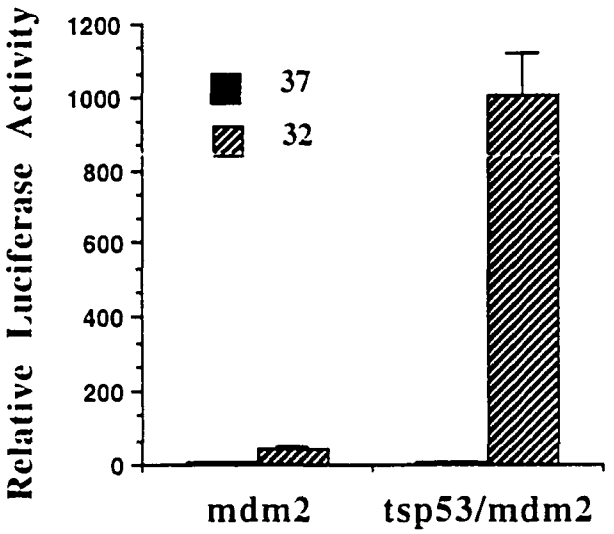
6.2 Clones from ZR75.1 cells transfected with the murine temperature sensitive p53 Val135. Cells were transfected with a plasmid expressing p53 Val135 and a plasmid expressing the Neomycin resistance gene or with the Neomycin resistance gene alone. Neomycin resistant clones were selected in media containing G418 (0.6mg/mL). Expression of p53 Val135 was monitored by western blot analysis of cell lysates with a mixture of p53 monoclonal antibodies.



6.3 Clones from MDA 468 cells transfected with the mdm2 gene
Cells were transfected with a plasmid expressing full length MDM2 (1A, 3B, 5A) or MDM2 lacking the p53 binding region (7A, 9A, 11A) and a plasmid expressing the Neomycin resistance gene. Neomycin resistant clones were selected in media containing G418 (0.3mg/mL). Expression of MDM2 was monitored by western blot analysis of cell lysates with MDM2 monoclonal antiserum (top). The level of p53 was also monitored by western blot analysis using a mixture of p53 monoclonal antibodies (bottom). 468 is the parental cell line, 7-4 is a neomycin resistant clone and 8-9 is a cloned expressing the temperature sensitive p53 Val 135.



6.4 Co-immunoprecipitation of MDM2 with p53 from lysates of cells stably transfected with the mdm2 plasmids. Lysates from the different clones were immunoprecipitated with the p53 antibody 1801 or 421. The presence of MDM2 in the precipitate was analyzed by Western blot analysis using an MDM2 monoclonal antiserum. WCL =whole cell lysates. No co-immunoprecipitation is detected but this gel is shown because it shows the potential MDM2 bands in the whole cell lysates.



6.5. Activity of the mdm2 reporter in MDA 468 cells in the presence or absence of temperature sensitive p53 Val 135. Cells were transfected with 10ug of the mdm2 reporter plasmid DNA and either 10 ug of carrier DNA or 10 ug of a plasmid expressing p53 Val 135. At 48 hours post-transfection, cells extracts were made from cells maintained at 37°C and cells switched to 32°C for 24 hours.

Chapter 7

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