BIOCHEMICAL INVESTIGATION OF PROTEIN/PROTEIN INTERACTIONS

INVOLVING ACID-RICH DOMAINS FROM TRANSCRIPTION FACTORS

by

TAMARA NICOLE JONES

(Under the Direction of Alan Przybyla)

ABSTRACT

In eukaryotic cells, transcription is regulated through a complex network of macromolecular interactions mediated by transcription factors, coactivators, and corepressors. To better understand how this network governs gene expression, it is critical to understand the precise details of the macromolecular interactions involved. The tumor suppressor protein p53 interacts specifically with several components of the general transcription machinery as well as numerous other cellular proteins. In the research presented here, nuclear magnetic resonance (NMR) and *in vitro* binding assays were used to examine the interaction between p53 and the cellular transcription factors TFIIH, CBP, and GATA. We have determined that p53 can bind to these three transcription factors through its amino-terminal acidic activation domain (AAD). Although all these factors bind to the same domain, it seems that they are each able to recognize distinct amino acids within the domain. Precise details like these are critical if we are to understand the intricacies of the transcription process.

INDEX WORDS: transcription; transactivation; p53; TFIIH; Tfb1; p62; CBP; GATA; acidic activation domain; AAD

BIOCHEMICAL INVESTIGATION OF PROTEIN/PROTEIN INTERACTIONS INVOLVING ACID-RICH DOMAINS FROM TRANSCRIPTION FACTORS

by

TAMARA NICOLE JONES

B.S., The University of Georgia, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

BIOCHEMICAL INVESTIGATION OF PROTEIN/PROTEIN INTERACTIONS

INVOLVING ACID-RICH DOMAINS FROM TRANSCRIPTION FACTORS

by

TAMARA NICOLE JONES

Major Professor: Alan Przybyla

Committee: Pascale Legault
James Omichinski
Michael Bender

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2004

DEDICATION

To Mom, Dad, and Valerie who have always encouraged me to pursue my dreams.

ACKNOWLEDGEMENTS

I would first like to acknowledge my major professors Drs. Jim Omichinski and Pascale Legault for seeing my potential and accepting me into their lab four years ago. I would especially like to thank Jim for introducing me to the wonderful world of protein biochemistry and his relentless reviewing and revising of my thesis. It is because of their guidance that I have grown as a person and scientist. In addition, I would also like to acknowledge the other members of my committee Drs. Alan Przybyla and Michael Bender. To my fellow lab members, Bao, Brian, Calvin, Dean, Greg, Hsin, Karen, Kris, Lisa, Paola, and Tom, I thank you for all of the laughs and encouragement. You made life as a graduate student a little easier. I would also like to acknowledge the newest additions to the department Dr. Jeff Urbauer , Ramona Urbauer, Josh, and Ryan for always being available for advice. A special thank you to my friends and family for all of your prayers and motivation.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
RNAPII	RNA Polymerase II
TBP	TATA-binding protein
TAF	TBP-associated factor
PIC	pre-initiation complex
HAT	histone acetyltransferase
AAD	acidic activation domain
HSF	heat shock factor
UAS_G	galactose upstream activating sequence
CD	circular dichroism
NMR	nuclear magnetic resonance
MDM2	mouse double minute 2 protein
Rb	retinoblastoma protein
VP16	herpes simplex viral protein 16
DBD	DNA-binding domain
RPA	replication protein A
Chk1 and Chk2	checkpoint kinase 1 and 2
MAP	mitogen-activated protein

JNK ..... Jun N-terminal kinase

PKC ..... protein kinase C

PCAF ..... p300/CBP-associated factor

HSQC ..... Heteronuclear Single Quantum Coherence

NR ..... nuclear hormone receptor

CBP ..... cylic AMP response element binding protein

IBiD ..... IRF-3 binding domain

ZBD ..... zinc-binding domain

NZ ..... amino-terminal ZBD

CZ ..... carboxyl-terminal ZBD

BA ..... basic arm

FOG-1 ..... Friend of GATA-1

ER ..... estrogen receptor

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In eukaryotic cells, RNA is synthesized by three classes of DNA-dependent RNA Polymerases (RNAP I, II, and III) [1]. Protein encoding genes are transcribed into messenger RNA (mRNA) by RNAPII. The synthesis of mRNA occurs in three stages: initiation, elongation, and termination (Figure 1.1) [2]. Initiation is important for the regulation of gene expression and includes the assembly of the preinitiation complex. Once assembled on the promoter, the polymerase along with general transcription factors are in an open complex formation and elongation begins [3]. Finally, transcription culminates with the release of the RNA transcript and a recycling of the polymerase and transcription factors.

Activators function by increasing transcription rates

Activators function by binding to specific DNA sequences and stimulating transcription through protein/protein interactions. Generally, activators contain two functional domains: a DNA-binding domain that recognizes a specific sequence in the target promoter and an activation domain that is required for interaction with other proteins involved in transcription regulation. In order to increase its concentration near the promoter, an activator is recruited by a very specific DNA sequence that can be from ten to tens of thousands of base pairs upstream of the regulated gene start site [4]. For high-level expression to occur, genes usually contain multiple copies of these regulatory DNA-binding sites. Once the activator contacts the specific site through its DNA binding domain, it is thought that the DNA loops out to allow interaction

between the activation domain and the general transcriptional machinery [5]. It has been shown that mutations in the DNA binding domain can inhibit the ability of the activator to stimulate gene expression [6, 7].

In addition to its DNA binding capability, activators have the ability to recruit the general transcriptional machinery to the promoter. Activators have been shown to interact with several general transcription factors including the TATA-binding protein (TBP) of IID (TFIID) [8-11], TBP-associated factors (TAFs) [12-14], IIB (TFIIB) [15-17], IIA (TFIIA) [4] and IIH (TFIIH) [18]. Interestingly, it appears that each of these interactions are crucial because mutations that inhibit these interactions also inhibit activator function [18-20]. Those that support the step-wise assembly model suggest that there are rate-limiting steps in preinitiation complex (PIC) assembly at the TATA-box binding site that are increased by activators. For example, it has been shown that the stable recruitment of general transcription factors TFIIB, TFIIF, TFIIE, and RNAPII were all increased in the presence of an activator [21]. On the other hand, many believe that the PIC exists as a holoenzyme complex consisting of TFIIA, TFIIB, TFIIF, TFIIH, with RNAPII and a SRB/Mediator subcomplex. Many activators have been shown to interact with the holoenzyme complex [22-26]. Identifying specific targets for activators within the PIC is a very tedious process since it consists of over 50 proteins. In addition to the extensive number of proteins involved, there is also the continuous debate of whether the PIC assembles in a step-wise fashion or exists in the cell as a holoenzyme complex. Although the exact mechanism is still unclear, the general consensus is that activators function by stabilizing PIC formation.

In addition to assembly of the PIC on the promoter, there are other barriers that must be overcome in order to enhance initiation. In the nucleus, DNA is compacted into a nucleoprotein complex called chromatin. Chromatin is comprised of repeating units of nucleosomes, which

contain approximately 150 base pairs of DNA wrapped around an octamer of core histone proteins. These nucleoprotein complexes have been demonstrated to have an inhibitory effect on transcription. Activators have been shown to help alleviate nucleosome mediated repression of transcription through interactions with chromatin remodeling complexes such as SAGA and SWI/SNF [27]. Histone acetyltransferases (HATs) within chromatin remodeling complexes function by acetylating lysines at the amino terminus of core histones [28]. This post-translational modification reduces the affinity of the core histone proteins for DNA and this leads to a destabilization of the chromatin complex. This destabilization of chromatin allows the transcriptional machinery to more efficiently access the DNA. Activators appear to target specific HAT complexes to nucleosomal DNA to acetylate the core-histone proteins and stimulate transcription [23, 29]. The SWI/SNF complex is a chromatin-remodeling enzyme that binds to the nucleosomal template and alters the twist of DNA in an ATP-dependent reaction that repositions the nucleosome [27]. It is believed that this repositioning of the nucleosome facilitates the binding of the general transcriptional machinery to its target. In particular, acidic activation domains (AAD) have been shown to specifically recruit the SWI/SNF complex to the DNA template. Finally, it appears that recruitment of the holoenzyme complex with RNAPII also promotes destabilization of chromatin by penetrating the nucleosome and directly competing with histones for DNA [30].

Activators function in many stages of transcription

It is well established that activators stimulate the transcription of genes, but it is now becoming increasingly clear that activators can function at several stages of the transcription process. The fact that activators stimulate transcription by recruiting general transcription

factors is known, but there is also compelling evidence that activators can function subsequent to promoter binding [31]. Research has shown that activators facilitate open complex formation. For example, when the activation domain of herpes simplex virus protein VP16 is mutated there is a decrease in open complex formation [32]. This decrease in open complex formation ultimately results in a decreased rate of transcription since this complex must be formed in order for the template to be transcribed. In addition to stimulating open complex formation, activators have also been shown to increase the rate of elongation. Various factors such as nucleotide depletion, RNA secondary structure, repressors and nucleosomes can cause what is known as promoter-proximal pausing. Activators are able to assist in reading through these pauses, through interaction with general transcription factors and/or chromatin remodeling enzymes. Heat shock factor (HSF), an activator that regulates the transcription of several genes in response to heat stress, has been used to study the effects activators have on elongation. *In vitro* GAL4 fusion proteins containing each of the activation domains (AD) of HSF were added to an elongation assay and resulted in an increase in transcription [33]. In addition, it was determined that bulky hydrophobic residues located in the AD were necessary for stimulating elongation. Other activators that have been shown to stimulate elongation include the HIV-1 Tat protein and VP16 [34, 35].

Classification of activators

Activation domains have been classified on the basis of prevalent amino acids in their primary sequence. This includes those that are acid-rich (AAD), glutamine-rich, serine/threonine (S/T)-rich as well as proline-rich activation domains. Thus far, AADs are the largest and most studied of this group and they include Gcn4, Gal4, p53, and VP16 [36-39]. Although the

remaining groups, glutamine-, S/T- and proline-rich, do not have as many representatives, respective examples include Sp1, TF3, and CTF [40].

As previously stated, in order for a protein to function as an activator it must contain two domains: a DNA-binding domain and an activation domain. This characteristic separated activators into two groups: the 'universal' activator and the 'non-universal' activator [41]. 'Universal' activators contain both a DNA-binding domain and an activation domain; therefore, it contains both components necessary for function. One example of a universal activator is the yeast regulatory protein Gal4. The DNA-binding domain of Gal4 interacts with a specific DNA sequence, the galactose upstream activating sequence or UAS_G. Once its DNA-binding domain makes contact, Gal4 is able to activate transcription through protein/protein interactions involving its AADs. On the other hand, 'non-universal' activators only contain one of the necessary domains and must interact with another protein that can provide the domain it lacks. VP16 is considered a 'non-universal' activator because it does not contain a DNA-binding domain. In order to activate transcription, it must interact with the octamer-motif binding factor Oct-1 [42]. Oct-1 contains the DNA-binding domain required for the activation of transcription by the AAD of VP16. Thus, it is a symbiotic relationship in that one protein cannot activate transcription without the presence of the other protein.

A third classification of activators is based upon what stage(s) in the transcriptional process the activator stimulates. Type I activators stimulate initiation only and include Sp1, CTF, as well as a mutant of VP16 called SW6. Type II activators are divided into two subgroups, type IIA and type IIB. Currently, there is one member of the type IIA subgroup, HIV Tat, which stimulates elongation only. The type IIB subgroup, however, stimulates both initiation and elongation. Its members include E2F1, and VP16. These conclusions were based

upon nuclear run-on, RNase protection, and reporter gene activity assays [43]. The validity of these classifications was verified when a mutation in VP16, SW6, changed it from a type II to a type I activator.

The final method of classification used is whether or not an activator can stimulate transcription at binding sites distal from the promoter. Various activators have been fused to the GAL4 DNA-binding domain and used to determine if they could activate transcription proximal or distal from the promoter [44]. It was shown that both acid-rich and S/T-rich activation domains could stimulate transcription from both proximal and distal positions, thereby, forming the 'general' group. This group included VP16, Gal4, p65, TFE3, ITF-1 and ITF-2. The 'proximal' group can only stimulate from a position close to the promoter. Examples of proximal activators include glutamine-rich activators such as Oct-1, Oct-2, and Sp1. Finally, the third group stimulates strongly near the promoter, but only weakly from remote sites. Members of this group include proline-rich activation domains AP-2 and CTF/NF1.

Structural studies on activation domains in the free form

The general consensus is that in a target free environment, activation domains exist in a highly flexible unstructured state. For example, researchers have described AADs, as being "acidic blobs" or "negative noodles" that adopt structure once they interact with a target protein [45]. In an attempt to clarify this theory, AADs from proteins such as VP16, GAL4, and p53 have been extensively studied in the free state.

Structural features of the VP16 AAD have been examined using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. It was first proposed that the AAD of VP16 exist as amphipathic α-helices in solution based on secondary structure prediction [46].

Further investigation using two-dimensional NMR experiments showed that the VP16 AAD was devoid of any secondary structure elements [47]. Therefore, the AAD of VP16 is thought to be unstructured in the free state.

Studies with other AADs have resulted in a variety of different conclusions. A synthetic peptide containing a portion of the AAD of p53 ($p53_{(14-28)}$) was reported to form two β-turns in the unbound state [48]. Results from multidimensional NMR experiments with the full-length AAD of p53 ($p53_{(1-73)}$) indicated that this domain exists as an amphipathic helix with two nascent turns [49]. However, a third study with this same region of p53 ($p53_{(1-73)}$) concluded that like the VP16 AAD, the p53 AAD is unstructured in the free form. In contrast, when the AAD of GAL4 was examined by genetic analysis, it was hypothesized that instead of an α-helix it may exist as a β-sheet in the free form [50]. Subsequent studies using CD spectroscopy supported this hypothesis. Experiments performed in an aqueous solution did not indicate the presence of an α-helical structure [51]. Instead, in both slightly acidic and neutral pH buffers, GAL4 appeared to have a β-sheet conformation. These examples exhibit various conformations including random coil, amphipathic α-helices, and β-sheets, demonstrating that generalizations about the structure of activation domains in the free form cannot be assumed. Some believe that this flexibility in structure allows activation domains to interact with various targets while still conferring specificity [49].

Structural studies on activation domains in complex

One similarity found with AADs is that they seem to adopt a more ordered secondary structure upon binding to a target protein. Complexes studied thus far include VP16 / $TAF_{II}31$,

p53 / MDM2, E2F-2 / Rb, and FCP / RAP74. The structures of these complexes have been determined using NMR spectroscopy and x-ray crystallography.

Mouse double minute 2 or MDM2, is a transcription factor that negatively regulates p53 by targeting it for degradation by the ubiquitin-protease pathway. The first example was the high-resolution structure of a complex containing a portion of the activation domain of the tumor suppressor p53 ($p53_{(13-29)}$) bound to the amino-terminal domain of MDM2 ($MDM2_{(13-118)}$). As previously stated, a synthetic peptide of free $p53_{(14-28)}$ is not highly structured only forming two β turns. Upon interaction with the amino-terminal domain of MDM2, residues 18 to 26 of p53 form an amphipathic α-helix of approximately 2.5 turns [52]. The highly conserved amino-terminal domain of MDM2 (amino acids 17-125) forms a cleft lined with numerous hydrophobic residues. This hydrophobic cleft contains four helices, making up the side and the bottom, and a pair of three-stranded β-sheets which act to close off the ends. Based on the crystal structure, it was determined that p53 interacts with MDM2 predominantly through hydrophobic interactions involving the cleft of MDM2 and hydrophobic amino acids in p53 especially Phe^{19}, Trp^{23}, and Leu^{26} [52]. These three residues of p53 insert deep into the cleft of MDM2 and make multiple van der Waals contacts with hydrophobic residues of MDM2. This hydrophobic interface between p53 and MDM2 was first proposed when mutation of Phe^{19}, Leu^{22} and Trp^{23} disrupted both the activation capabilities of p53 and its interaction with MDM2 [53]. In addition to mutational analysis, the fact that amino acids 13-23 of p53 are conserved in a number of species also emphasizes the importance of this interaction.

As with the p53/MDM2 complex, VP16 forms an amphipathic α-helix following binding to $hTAF_{II}31$ [54]. The interface of this complex was determined using the amino-terminus of $hTAF_{II}31$ (a.a. 1-181) and the carboxy-terminus of VP16 (a.a. 452-490). The precise interactions

were defined using two-dimensional NMR spectroscopy. The studies revealed that amino acids Phe^{479} and Leu^{483} make hydrophobic interactions while Asp^{472} makes salt-bridge contacts with $hTAF_{II}31$. In order to verify these results, mutations of Phe^{479} and Leu^{483} in VP16 were made resulting in a loss of binding to $hTAF_{II}31$ and decrease in transactivation activity [54]. These critical residues in VP16 resemble the pattern of hydrophobic residues found in the α-helix of p53 in the p53/MDM2 complex.

Another recently determined structure was a complex between the activation domain of transcription factor E2F and the retinoblastoma (Rb) tumor suppressor protein. In the cell, Rb interacts with E2F and represses its ability to activate transcription [55]. Previous studies have shown that the pocket region of Rb is essential for its interaction with E2F. This pocket in Rb consists of three domains referred to as the A-box, the B-box and the carboxyl-terminal tail and includes residues 360-581 and 643-785. The two segments are joined together by a highly flexible linker that can be removed without destroying the interaction. The region of E2F-2 essential for binding to Rb consists of 18 residues (410-427) within its acidic carboxyl-terminal activation domain. This region in the E2F family (E2F1-6) is highly conserved with 10 of the 18 residues being identical in all family members. A complex between E2F-2 and Rb has been studied using x-ray crystallography [56]. Amino acids 410-416 of E2F-2 interact with polar and hydrophobic residues of the A-box and B-box of Rb through hydrogen bonds and van der Waals interactions forming a β-turn. Key residues for contact include Tyr^{411} and Phe^{413}, and mutation of these amino acids in the highly homologous E2F-1 has been shown to disrupt binding [56, 57]. The carboxyl-terminal segment (amino acids 422-427) of E2F-2 forms an α-helix that interacts with the A-box of Rb through hydrogen bonding and van der Waals interactions. This was in agreement with mutational analysis of E2F-1 that has demonstrated that residues in the

carboxyl-terminal segment (Ile^{422}, Leu^{425}, and Phe^{426}) are critical for interaction [56, 57]. The crystal structure of E2F-2 / Rb revealed that the function of Rb is most likely to mask residues necessary for transcriptional activation.

Finally, multidimensional NMR and x-ray crystallography have been used to solve a complex between the AAD of the RNAPII CTD phosphatase FCP1, and a subunit of transcription factor TFIIF, RAP74. Like other AADs, FCP1 (residues 837-961) goes from an unstructured form to an amphipathic α-helix upon interaction with its target RAP74 (residues 436-517). The binding surface for $FCP1_{(837-961)}$ on $RAP74_{(436-517)}$ consists of hydrophobic and basic residues that form a shallow groove between two α-helices (H2 and H3) [58]. This groove allows $RAP74_{(436-517)}$ to contact the amphipathic α-helix formed by $FCP1_{(837-961)}$ (H1) through intermolecular salt bridges as well as polar and van der Waals interactions. These interactions involve five highly conserved acidic side chains from $FCP1_{(837-961)}$, Glu^{945}, Asp^{947}, Glu^{954}, Glu^{956}, and Asp^{959}, and all are required for binding. A consensus binding sequence, L/IEXELXDhh, was postulated based upon analysis of the solution structure of the $FCP1_{(837-961)}$ / $RAP74_{(436-517)}$ [58]. This motif in FCP1 is very similar to the LXXLL and LXXI/HIXXXL/I motifs found in nuclear hormone receptor (NR) coactivators and NR corepressor proteins respectively [59, 60]. In addition to the NMR solution structure of the FCP1 / RAP74 complex there is also a nearly identical x-ray crystal structure of a complex of $FCP1_{(941-961)}$ and $RAP74_{(451-517)}$ [61].

Overview of structural studies on activation domains

There is now enough information from structural studies of AADs in the free and the bound state to make a few general conclusions. The general consensus is that in the free form, AADs do not appear to be highly structured. Each of these complexes discussed are similar in

that the AAD is unstructured in the free form but adopts a more ordered structure upon complex formation. In addition, hydrophobic residues within the AAD are essential for binding. This is consistent with previous mutational analysis that demonstrated the importance of aromatic and hydrophobic amino acids in these domains in addition to the acidic residues [53, 54]. In terms of the target proteins, MDM2, Rb, and RAP74 all form a pocket to accommodate the helices formed by the AADs. These pockets consist of crucial hydrophobic residues required for interaction, but in the case of Rb and RAP74 there are also numerous polar interactions. Interestingly, there are very few polar interactions in the p53/MDM2 complex despite the large number of acidic residues in the AAD of p53. It is also interesting to note that while some structural elements are the same in each target protein, each complex is unique in terms of their overall three-dimensional fold. This uniqueness in the targets of activation domains may aid in conferring the specificity of these protein/protein interactions.

p53: a cellular acidic activator

Eukaryotic cells are constantly subjected to both external and internal stresses such as ultra-violet light, chemicals, and DNA damage. In order to combat these agents, the cell has checkpoints used to trigger pathways that lead to either DNA repair or cell death (apoptosis). When these checkpoints detect DNA damage it slows cell cycle progression in order to prevent DNA replication, and allows time for repair mechanisms to be activated. In the event the DNA damage cannot be repaired, apoptosis occurs. These cellular responses are essential to preserve the integrity of the information passed to daughter cells. Tumor suppressor p53 plays an important part in this process. In response to stress, p53 has a major role in both growth arrest and apoptosis. Increased interest in p53 developed when it was determined that mutations in its

gene are in over 50% of all human tumors. Defining the regulation, modifications, and interactions of p53 are important for understanding how it reduces the incidence of cancers.

Domains and domain function

Human p53 consists of 393 amino acid residues, and it can be divided into several functional domains (Figure 1.2). The highly acidic amino-terminus contains a transcriptional activation domain encompassing amino acids 1-63 [62-64]. This region is considered a typical AAD and contains predominantly acidic and hydrophobic amino acid residues. Adjacent to the AAD is the proline rich domain, residues 64-91, containing 12 prolines. Similar to other proteins involved in signal transduction pathways, this proline-rich domain contains five repeats of the PXXP motif. This PXXP motif is highly conserved, and it is thought to function in transmitting antiproliferative signals to induce apoptosis [65]. This domain is not required for transcriptional activation, however it is required for p53 to suppress growth of tumorigenic cells. The core domain (amino acids 102-292 in human p53) contains sequence specific DNA-binding activity. This DNA-binding domain (DBD) recognizes the DNA consensus sequence 5'-PuPuPuC(A/T)(T/A)GpyPyPy-3'. It is in the DBD where the majority of mutations occur in humans leading to tumor formation. These mutations disrupt p53 function by either preventing DNA binding or by affecting the proper folding of the protein [66]. In addition to the DBD, mutations in the nuclear localization domain (amino acids 316-325) are also observed in many human cancers. Mutations in this domain cause tumors to occur because after DNA damage is recognized, p53 is unable to enter the nucleus where it can interact with transcriptional machinery [67]. Therefore, p53 is unable to suppress tumor activity by inducing apoptotic response. In the cell, p53 exists as a tetramer formed by residues 334-355. This oligomerization

domain is separated from the DNA-binding domain by a flexible linker of 37 residues [68, 69]. The last 29 amino acids of the carboxy terminus contain a regulatory domain, which is able to negatively regulate the sequence specific DNA-binding ability of the core domain [70]. This region is highly basic, protease sensitive, and interacts with RNA and DNA [71]. Each of these domains has an important role in the function of p53 and collectively regulates its response to DNA damage.

Initially, the AAD was mapped within the first 40 amino acids (AAD1). This fragment when fused to a GAL4 DBD activated transcription in human lung cancer cells [72]. These first forty amino acids are essential for protein/protein interactions with several p53 targets including MDM2, $TAF_{II}40$, $TAF_{II}31$, and the KIX domain of CREB binding protein (CBP) [14, 54, 73, 74]. Interestingly, residues 13-23 are highly conserved in a number of species and a mutation within these 11 amino acids inhibits binding to a number of target proteins. Further investigation revealed a second activation domain between amino acids 40 and 63. It was shown when fused to a GAL4 DBD, amino acids 40-83 (AAD2) of p53 not only activated transcription but was as potent as residues 1-40 (AAD1) [62]. It was later determined that AAD2 targets several proteins including TBP, replication protein A (RPA), and the SRC-1 domain of CBP/p300 [75-77]. Despite binding different targets, it has been shown that the activity of the fragment containing both subdomains, $p53_{(1-87)}$, was more potent than the sum of both AAD1 ($p53_{(1-40)}$) and AAD2 ($p53_{(40-83)}$) [62]. Both AAD1 and AAD2 are similar in amino acid composition containing numerous acidic and hydrophobic residues. It is interesting to note that several other activator proteins (i.e. VP16) contain an AAD consisting of two or more subdomains with synergistic activity [78]. Investigating why targets interact with AAD1, AAD2, or both and how this

interaction contributes to the function of p53 will help determine how the activation of transcription by p53 is regulated.

Response to upstream events

Found in the cytoplasm of unstressed cells, p53 is normally a short-lived protein degraded after approximately 20 minutes [79]. Due to this short half-life, the concentration of p53 is generally kept very low. This inactive form is maintained in part by MDM2, a transcription factor that negatively regulates p53 by inhibiting its transcriptional activity and targeting it for degradation by the ubiquitin-protease pathway [80, 81].

There are several different cellular events that can trigger a cascade of responses that convert p53 to its active form (Figure 1.3a). For example, single and double stranded DNA breaks caused by ionizing radiation, such as γ- or ultraviolet-radiation, are detected by proteins which examine chromatin for malformations [82]. Once found, signal transduction pathways are then activated which in turn report DNA damage to the proper repair machinery. The presence of these DNA repair intermediates can also elicit a response. Another cellular event leading to p53 activation is nucleotide depletion. It has been shown that CTP, GTP, or UTP depletion caused the arrest of cell cycle progression induced by p53 [83]. In order for DNA replication to occur these nucleotides must be available, therefore when concentrations fall below a critical level, cell-cycle arrest allows the depleted cell to replenish nucleotide concentrations. A third type of signal that leads to p53 activation is low levels of oxygen or hypoxia. Used as a defense mechanism in tumorigenic cells, hypoxia occurs in order to induce apoptosis preventing tumor development. Hypoxia causes an increase in the level of p53 while decreasing the presence of its inhibitors that would prevent cell death thereby promoting tumor formation [84].

Post-translational modifications: phosphorylation and acetylation

Post-translational modifications play a vital role in regulating the stability and activity of p53 in cells (Figure 1.3b). These modifications occur primarily at the amino- and carboxyl-terminal domains of the protein. The amino-terminal domain contains residues that affect protein/protein interactions and transcriptional activation, whereas the carboxyl-terminal domain plays a role in regulating sequence specific DNA-binding. Modified on several amino acid residues, phosphorylation and acetylation of residues of p53 are essential in order for proper functioning.

p53 has been shown to be phosphorylated on at least 12 different amino acids with many occurring in the amino-terminal activation domain (Figure 1.4). Phosphorylation by casein kinase I has been reported on Ser^{6} and Ser^{9} in response to UV radiation [85]. One of the most critical residues, Ser^{15}, is typically phosphorylated after DNA damage by DNA-PK [86]. This modification inhibits interaction of p53 with MDM2 and TFIID, but increases the binding of p53 with CBP/p300 [86-88]. Thr^{18} can be phosphorylated by casein kinase I while Ser^{20} is phosphorylated by checkpoint kinase 1 and 2 (Chk1 and Chk2) [89, 90]. Phosphorylation of either of these residues contributes to p53 regulation. This is accomplished differently by each residue. Thr^{18} affects the interaction between p53 and MDM2 and Ser^{20} regulating the turnover rate of the protein [89, 91]. The final four additional residues in the amino-terminal domain that are phosphorylated in response to UV light include Ser^{33} and Ser^{46} which are phosphorylated by mitogen-activated protein (MAP) kinase, Thr^{81} which is phosphorylated by Jun N-terminal kinase (JNK) and Ser^{37} phosphorylated by DNA-PK [87, 92, 93]. Additional sites of phosphorylation occur in the carboxyl-terminal domain of the protein. Ser^{315} and Ser^{392} are

phosphorylated by cdk2/cdc2 and casein kinase II respectively in response to UV radiation and Ser^{378} is phosphorylated by protein kinase C (PKC) [94, 95].

Acetylation is another post-translational modification of p53 seen in response to DNA damage or UV radiation. Acetylation contributes to the stabilization and activity of p53 by increasing its half-life. In addition, acetylation appears to serve a role in cell cycle arrest at the G_1 stage, and activates p53 sequence-specific DNA-binding activity [96-98]. This acetylation occurs in the carboxyl-terminal domain, particularly in the regulatory domain (Figure 1.4). Lysine acetylation is achieved typically by one of two acetyltransferases, p300/CBP or p300/CBP associated factor (PCAF). In response to DNA damage, p300/CBP acetylates Lys^{373}, Lys^{381}, Lys^{382} *in vitro* and *in vivo* [98, 99]. Lastly, PCAF acetylates Lys^{320} and this also results in activating DNA-binding activity [100].

Role of p53 in tumorigenesis

The activation of p53 typically results in one of two outcomes: cell cycle arrest or apoptosis (Figure 1.3c). These mechanisms are necessary in order to prevent DNA damage from being replicated in S phase of the cell cycle and transferred to daughter cells. It is interesting to note that the same events can lead to cell-cycle arrest in some cells and apoptosis in other cells. Clarifying which pathway is chosen and why is a difficult process due to the number of variables that must be considered. These variables include amongst other things, the degree of DNA damage and the level of p53 in the cell.

If there is a possibility that damage in the cell can be corrected, p53 serves to activate the appropriate genes to cause cell cycle arrest in order to allow time for DNA repair. This is typically done by either inducing or repressing genes that will cause this arrest early in the cell

cycle or G_1 phase. For example, p21, an inhibitor of cyclin dependent kinases and precursor to p53-dependent G_1 arrest, is upregulated by p53 [101]. It has been shown that during nucleotide depletion there is a correlation between arrest and the induction of p53 and p21 [83]. p21 is merely one of over 150 genes activated by p53 in response to cellular damage. In addition to nucleotide depletion other events have been shown to cause cell cycle arrest including exposure to γ-radiation and nucleotide biosynthesis inhibitors [83, 102]. If the damage in the cell is severe, a viral or cellular oncogene is present, or there is little to no expression of a tumor suppressor, apoptosis is the pathway chosen to prevent DNA replication. In contrast to its role in cell cycle arrest, p53 does not have to function as a transcription factor in order to trigger apoptosis [102]. However, there are genes that are regulated by p53 that can affect the apoptotic pathway [79]. This pathway is often activated during viral infections. For example, when the adenovirus E1B protein is induced in the cell, it is able to inhibit p53-dependent apoptosis. Bax, a protein upregulated by p53, interacts with E1B and prevents it from altering this pathway [103]. Other viral events that have been shown to elicit p53-mediated apoptosis include expression of the adenovirus E1A protein or human papilloma virus E7 protein [104, 105].

Structural studies of p53 using NMR and x-ray crystallography

Structural studies of transcriptional activators have been conducted in an attempt to help understand their functions better. Both NMR and x-ray crystallography have been used to characterize various domains of p53. The sequence-specific DNA-binding domain (DBD) of human p53 ($p53_{(102-292)}$) contains a beta sandwich with two loops and a loop-sheet-helix motif when complexed with DNA [66]. This same fold is also seen in the crystal structure of the free DBD of mouse $p53_{(92-292)}$ [106]. The oligomerization domain of p53 ($p53_{(319-360)}$) has also been

well characterized by NMR and x-ray crystallography. This domain is a tetrameric structure consisting of a dimer of dimers that is stabilized by hydrophobic and electrostatic interactions between the monomeric and dimeric units [69, 107].

Of particular interest to us are the structures of the AAD of p53. Like other AADs such as VP16 and GAL4, the activation domain of p53 is unstructured in the free state. These regions described as "acidic blobs" or "negative noodles" typically adopt a more ordered structure following interaction with a target protein [45]. For example, synthetic peptides encompassing $p53_{(14-28)}$ as well as $p53_{(1-73)}$ were shown to form two β-turns and two nascent turns respectively in the unbound state [48, 49]. As mentioned above, $p53_{(14-28)}$ adopts a more ordered secondary structure upon interacting with the target protein MDM2 (Figure 1.5) [52]. Residues 18 to 26 form an amphipathic α-helix of approximately 2.5 turns, which interacts with the hydrophobic cleft formed by residues 17 to 125 of MDM2. This highly conserved cleft in MDM2 consists of four helices making up the sides and bottom with a pair of three-stranded β-sheets closing off the ends. Hydrophobic and aromatic residues (Phe^{19}, Trp^{23}, and Leu^{26}) of p53 interact with MDM2 by inserting deep into its cleft and makes multiple van der Waals contacts with MDM2. Mutations of this hydrophobic interface disrupt the ability of p53 to interact with MDM2 (Lin 1995). $p53_{(14-28)}$ is located within the first activation domain of p53 (AAD1). Currently, there are no structural studies available of a complex containing either AAD2 or the full activation domain ($p53_{(1-73)}$).

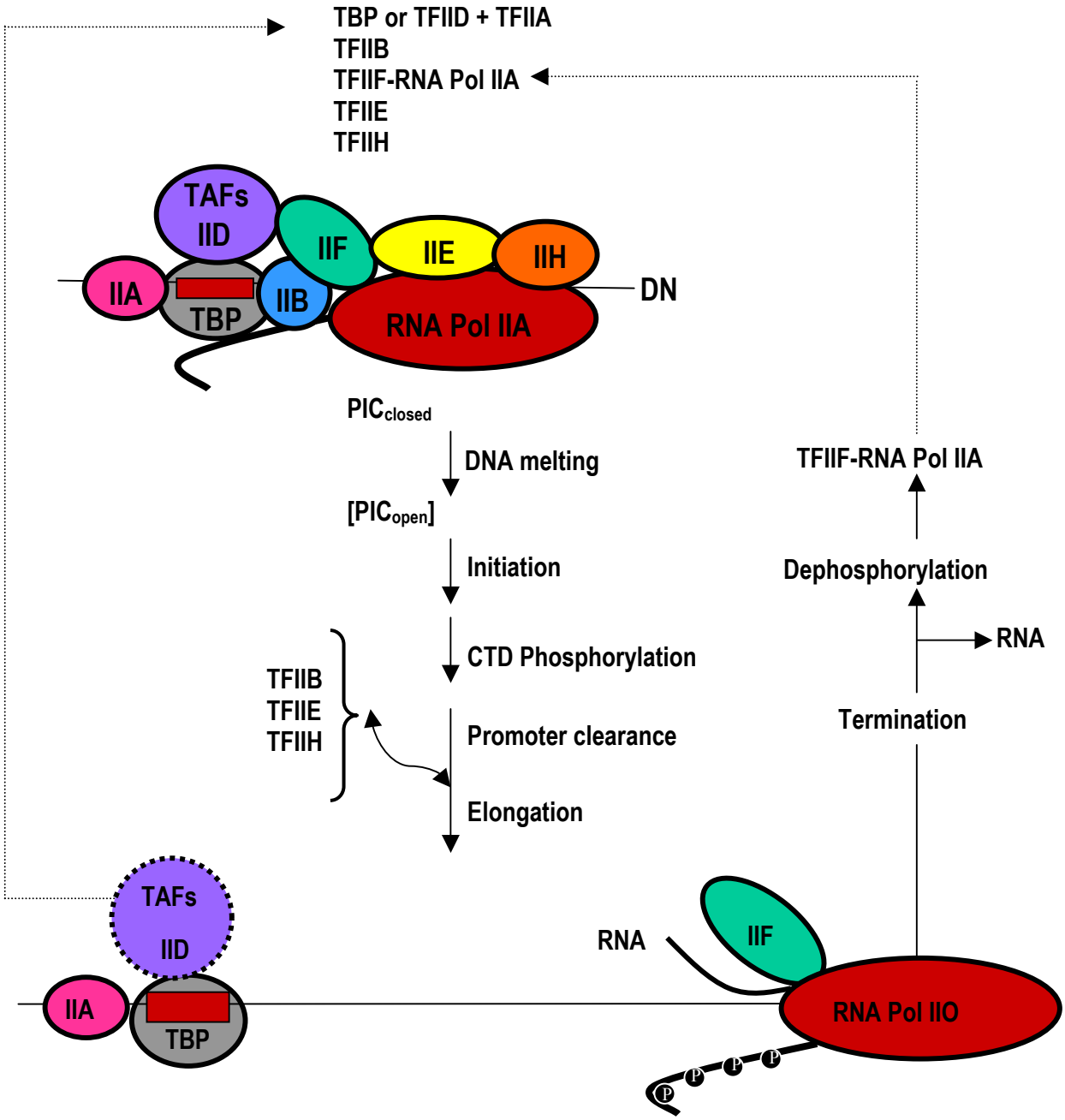


Figure 1.1. Model of eukaryotic transcription. In the step-wise assembly model general transcription factors along with RNA Pol II assemble on the promoter of a gene forming the pre-initiation complex (PIC). Following phosphorylation of the carboxyl-terminal domain (CTD) of RNA Pol II, elongation of the transcript begins culminating with termination and a recycling of the polymerase (Adapted from Roeder, 1996).

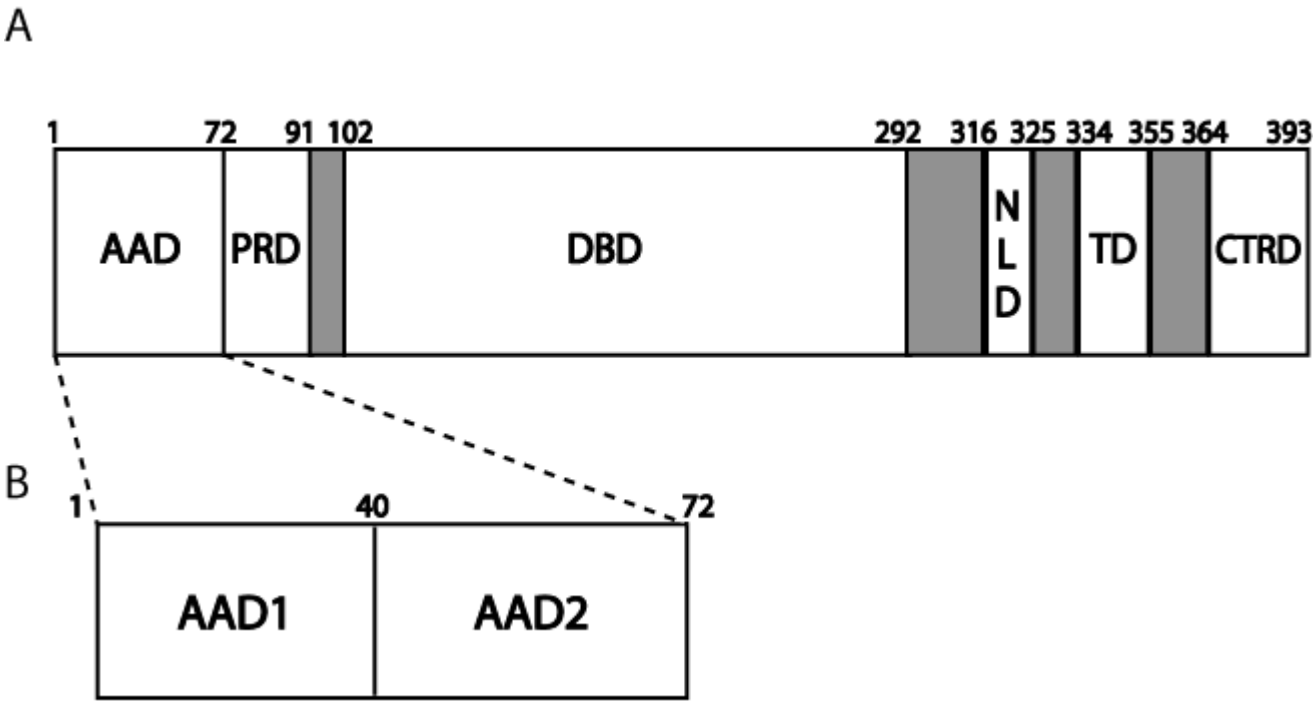


Figure 1.2. Schematic representation of human p53. (A) Regions associated with acidic activation domain (AAD), proline-rich domain (PRD), DNA binding domain (DBD), nuclear localization (NLD), tetramerization (TD) and a carboxyl-terminal regulatory domain (CTRD). (B) The TAD domain can be divided into two subdomains (AAD1 and AAD2).

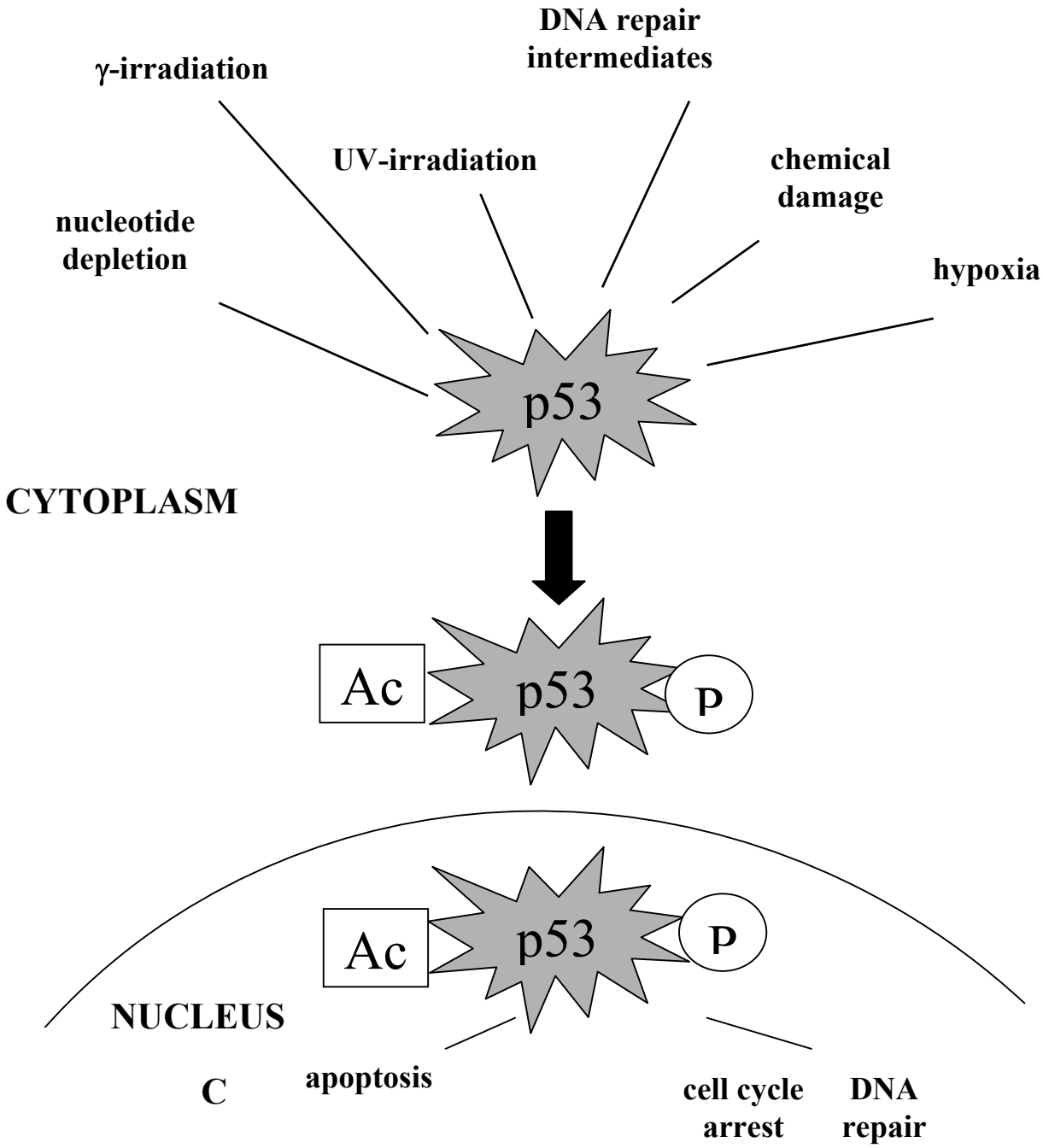


Figure 1.3. DNA damage induces a cascade of responses mediated by p53. (A) Upstream events resulting in DNA damage elicits the activation of p53. (B) Post-translational modifications including phosphorylation and acetylation stabilize p53 where it accumulates in the nucleus (C) Based on extent of DNA damage p53 activates transcription of downstream genes responsible for cell cycle arrest, DNA repair, or apoptosis.

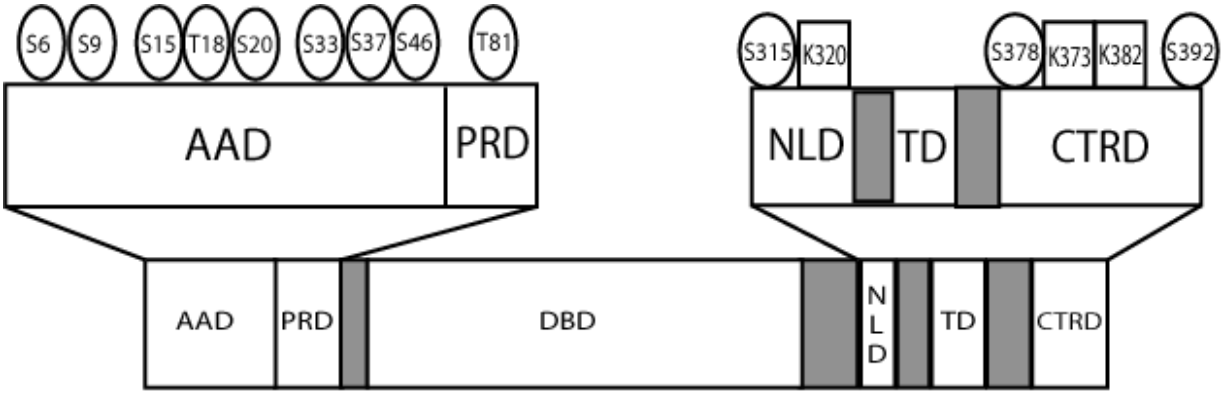


Figure 1.4. Post-translational modifications of p53. The residues of known sites of phosphorylation (0) and acetylation (□) induced by DNA damage.

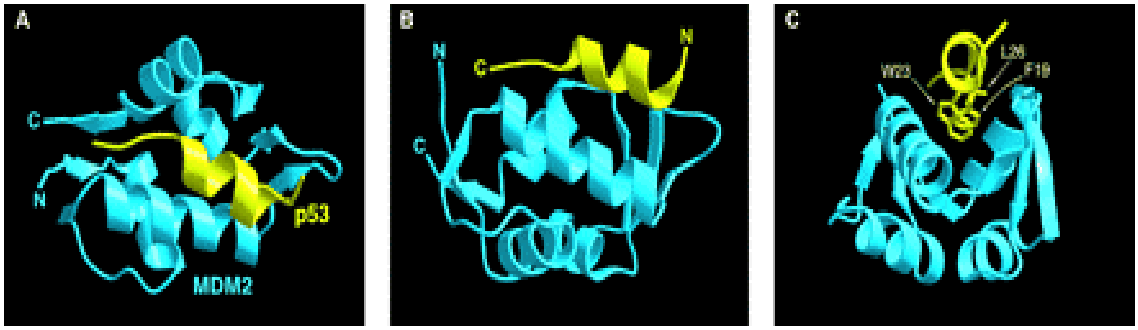


Figure 1.5. Ribbon diagram of MDM2$_{(13-118)}$ in complex with p53$_{(13-29)}$. The amino-terminal domain of MDM2 (cyan) forms a trough lined with hydrophobic residues. In this hydrophobic cleft, p53 (yellow) binds as an amphipathic α-helix (Adapted from Kussie, 1996).

References

1. Archambault, J. and J. Friesen, *Genetics of eukaryotic RNA polymerase I, II, and III.* Microbiol. Rev., 1993. **57**: p. 703-724.

2. Orphanides, G., T. Lagrange, and D. Reinberg, *The general transcription factors of RNA polymerase II.* Genes & Dev., 1996. **10**(21): p. 2657-83.

3. Lu, H., et al., *The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex.* Proc. Natl. Acad. Sci. USA., 1991. **88**: p. 10004-10008.

4. Kobayashi, N., T. Boyer, and A. Berk, *A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-Promoter Complex assembly.* Mol. Cell. Biol., 1995: p. 6465-6473.

5. Ptashne, M., *Gene regulation by proteins acting nearby and at a distance.* Nature, 1986. **322**: p. 697-701.

6. Tyler, J., K. Allen, and R. Everett, *Mutation of a single lysine residue severely impairs the DNA recognition and regulatory functions of the VZV gene 62 transactivator protein.* Nucl. Acids Res., 1994. **22**: p. 270-278.

7. Pissios, P., et al., *SREBP-1 binds to multiple sites and transactivates the human ApoA-II promoter in vitro.* Arterioscler. Thromb. Vasc. Biol., 1999. **19**: p. 1456-1469.

8. Stringer, K., C. Ingles, and J. Greenblatt, *Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID.* Nature, 1990. **345**: p. 783-786.

9. Geisberg, J., et al., *The zinc finger region of the E1A transactivating domain complexes with the TATA box-binding protein.* Proc. Natl. Acad. Sci. USA, 1994. **91**: p. 192-196.

10. Horikoshi, N., et al., *Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor TFIID.* Proc. Natl. Acad. Sci. USA, 1991. **88**: p. 5124-5128.

11. Horikoshi, N., et al., *Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression.* Mol. Cell. Biol., 1995. **15**: p. 227-234.

12. Goodrich, J., et al., *Drosophila $TAF_{II}40$ interacts with both a VP16 activation domain and basal transcription factor TFIIB.* Cell, 1993. **75**: p. 519-530.

13. Gill, G., et al., *A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the $dTAF_{II}IIO$ component of the Drosophilia TFIID complex and mediates transcriptional activation.* Proc. Natl. Acad. Sci. USA, 1994. **91**: p. 192-196.

14. Thut, C., et al., *p53 transcriptional activaion mediated by coactivators $TAF_{II}40$ and $TAF_{II}60$.* Science, 1995. **267**: p. 100-104.

15. Kim, T. and R. Roeder, *Proline-rich activator CTF-1 targets the TFIIB assembly step during transcriptional activation.* Proc. Natl. Acad. Sci. USA, 1994. **91**: p. 4170-4174.

16. Lin, Y., et al., *Binding of general transcription factor TFIIB to an acidic activating region.* Nature, 1991. **353**: p. 569-571.

17. Xu, X., et al., *Functional interaction of the v-Rel and c-Rel oncoprotein with the TATA-binding and association with transcription factor IIB.* Mol. Cell. Biol., 1993. **13**: p. 6733-6741.

18. Xiao, H., et al., *Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53.* Mol. Cell. Biol., 1994. **14**: p. 7013-7024.

19. Ingles, C., et al., *Reduced binding of TFIID to transcriptionally compromised mutants of VP16.* Nature, 1991. **351**: p. 588-590.

20. Roberts, S., et al., *Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation.* Nature, 1993. **363**: p. 741-744.

21. Choy, B. and M. Green, *Eukaryotic activators function during multiple steps of preinitiation complex assembly.* Nature, 1993. **366**: p. 531-536.

22. Hengartner, C., et al., *Association of an activator with an RNA polymerase II holoenzyme.* Genes & Dev., 1995. **9**: p. 897-910.

23. Drysdale, C., et al., *The Gcn4p activation domain interacts specifically in vitro with RNA polymerase II holoenzyme, TFIID, and the Adap-Gcn5p coactivator complex.* Mol. Cell. Biol., 1998. **18**: p. 1711-1724.

24. Natarajan, K., et al., *Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/Mediator.* Mol. Cell, 1999. **4**: p. 657-664.

25. Koh, S., et al., *An activator target in the RNA polymerase II holoenzyme.* Mol. Cell, 1998. **1**: p. 895-904.

26. Lee, Y., et al., *An activator binding module of yeast RNA polymerase II holoenzyme.* Mol. Cell. Biol., 1999. **19**: p. 2967-2976.

27. Neely, K., et al., *Transcription activator interactions with multiple SWI/SNF subunits.* Mol. Cell. Biol., 2002. **22**: p. 1615-1625.

28. Becker, P. and W. Horz, *ATP dependent nucleosome remodeling.* Annu. Rev. Biochem., 2002. **71**: p. 247-273.

29. Utley, R., et al., *Transcriptional activators direct histone acetyltransferase complexes to nucleosomes.* Nature, 1998. **394**: p. 498-501.

30. Ptashne, M. and A. Gann, *Transcriptional activation by recruitment.* Nature, 1997. **386**: p. 569-577.

31. Yankulov, K., et al., *Transcriptional elongation by RNA polymerase II is stimulated by transactivators.* Cell, 1994. **77**: p. 749-759.

32. Jiang, Y., S. Triezenberg, and J. Gralla, *Defective transcriptional activation by diverse VP16 mutants associated with a common inablility to form open promoter complexes.* J. Biol. Chem., 1993. **269**: p. 5505-5508.

33. Brown, S., et al., *Transcriptional activation domains stimulate initiation and elongation at different times and via different residues.* EMBO J., 1998. **17**: p. 3146-3154.

34. Bentley, D., *Regulation of transcriptional elongation by RNA polymerase II.* Curr. Opin. Genes & Dev., 1995. **5**: p. 210-216.

35. Greenblatt, J., J. Nodwell, and S. Mason, *Transcriptional antitermination.* Nature, 1993. **364**: p. 401-406.

36. Hope, I. and K. Struhl, *Functional dissection of a eukaryotic transcriptional activator protein, Gcn4 of yeast.* Cell, 1986. **46**: p. 885-894.

37. Ma, J. and M. Ptashne, *Deletion analysis of Gal4 defines two transcriptional activating segments.* Cell, 1987. **48**: p. 847-853.

38. Fields, S. and S. Jang, *Presence of a potent transcriptional activating sequence in the p53 protein.* Science, 1990. **249**: p. 1046-1049.

39. Triezenberg, S., R. Kingsbury, and S. McKnight, *Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early expression.* Genes & Dev., 1988. **2**: p. 718-729.

40. Mitchell, P. and R. Tjian, *Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins.* Science, 1989. **245**: p. 371-378.

41. Ptashne, M. and A. Gann, *Activators and targets.* Nature, 1990. **346**: p. 329-331.

42. Stern, S., M. Tanaka, and W. Herr, *The oct-1 homeodomain directs formation of a multiprotein DNA complex with the HSV transactivator VP16.* Nature, 1989. **341**: p. 783-786.

43. Blau, J., et al., *Three functional classes of transcriptional activation domains.* Mol. Cell. Biol., 1996. **16**: p. 2044-2055.

44. Seipel, K., O. Georgiev, and W. Shaffner, *Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions.* EMBO J., 1992. **13**: p. 4961-4968.

45. Sigler, P., *Acid blobs and negative noodles.* Nature, 1988. **333**: p. 210-212.

46. Reiger, J., F. Shen, and S. Triezenberg, *Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator.* Proc. Natl. Acad. Sci. USA, 1993. **90**: p. 883-887.

47. O'Hare, P. and S. Williams, *Structural studies of the acidic transactivation domain of the Vmw65 protein of herpes simplex virus using ^{1}H NMR.* Biochem., 1992. **31**: p. 4150-4156.

48. Botuyan, M., J. Momand, and Y. Chen, *Solution conformation of an essential region of the p53 transactivation domain.* Folding Des., 1997. **2**: p. 331-342.

49. Lee, H., et al., *Local structural elements in the mostly unstructured transcriptional activation domain of human p53.* J. Biol. Chem., 2000. **275**: p. 29426-29432.

50. Leuther, K., J. Salmeron, and S. Johnston, *Genetic evidence that an activation domain of GAL4 does not require acidity and may form a beta sheet.* Cell, 1993. **72**: p. 575-585.

51. Van Hoy, M., et al., *The acidic activation domains of the GCN4 and GAL4 proteins not alpha helical but form beta sheets.* Cell, 1993. **72**: p. 587-594.

52. Kussie, P., et al., *Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain.* Science, 1996. **274**: p. 948-953.

53. Lin, J., et al., *Functions of the p53 protein in growth regulation and tumor suppression.* Cold Spring Harbor Symposia on Quantitative Biology, 1995: p. 215-223.

54. Uesugi, M., et al., *Induced α-helix in the VP16 activation domain upon binding to a human TAF.* Science, 1997. **277**: p. 1310-1313.

55. Flemington, E., S. Speck, and W. Kaelin, *E2F-1 mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product.* Proc. Natl. Acad. Sci. USA, 1993. **90**: p. 6914-6918.

56. Lee, C., et al., *Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor.* Genes & Dev., 2002. **16**: p. 3199-3212.

57. Shan, B., T. Durfee, and W. Lee, *Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis.* Proc. Natl. Acad. Sci. USA, 1996. **9**: p. 679-684.

58. Nguyen, B., et al., *NMR structure of a complex containing the TFIIF subunit RAP74 and the RNA polymerase II carboxyl-terminal domain phosphatase FCP1.* Proc. Natl. Acad. Sci. USA, 2003, **100**: p. 5688-5693.

59. Heery, D., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**: p. 733-736.

60. Hu, X. and M. Lazar, *The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors.* Nature, 1999. **402**: p. 93-96.

61. Kamada, K., R. Roeder, and S. Burley, *Molecular mechanism of recruitment of TFIIF-associating RNA polymerase C-terminal domain phosphatase (FCP1) by transcription factor IIF.* P Proc. Natl. Acad. Sci. USA, **100**: p. 2296-2299.

62. Candau, R., et al., *Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity.* Oncogene, 1997. **12**: p. 807-816.

63. Zhu, J., et al., *Identification of a novel p53 functional domain that is necessary for mediating apoptosis.* J. Biol. Chem., 1998. **273**: p. 13030-13036.

64. Venot, C., et al., *Definition of a p53 transactivation function--deficient mutant and characterization of two independent p53 transactivation subdomains.* Oncogene, 1999. **18**: p. 2405-2410.

65. Walker, K. and A. Levine, *Identification of a novel p53 functional domain that is necessary for efficient growth suppression.* Proc. Natl. Acad. Sci. USA, 1996. **93**: p. 15335-15340.

66. Cho, Y., et al., *Crystal structure of a p53 tumor suppressor--DNA complex: understanding tumorigenic mutations.* Science, 1994. **265**: p. 346-355.

67. Shaulsky, G., et al., *Nuclear localization is essential for the activity of p53 protein.* Oncogene, 1991. **6**(11): p. 2055-65.

68. Struzbecher, H., et al., *A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization.* Oncogene, 1992. **8**: p. 1513-1523.

69. Jeffrey, P.D., S. Gorina, and N.P. Pavletich, *Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms.* Science, 1995. **267**(5203): p. 1498-502.

70. Ko, L.J. and C. Prives, *p53: puzzle and paradigm.* Genes & Dev., 1996. **10**(9): p. 1054-72.

71. Lee, S., et al., *p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches.* Cell, 1995. **81**(7): p. 1013-20.

72. Unger, T., et al., *p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer.* EMBO J., 1992. **11**(4): p. 1383-90.

73. Oliner, J.D., et al., *Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53.* Nature, 1993. **362**(6423): p. 857-60.

74. Van Orden, K., et al., *Binding of p53 to the KIX domain of CREB binding protein.* J. Biol. Chem., 1999. **274**: p. 26321-26328.

75. Liu, X., et al., *The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription.* Mol. Cell. Biol., 1993. **13**(6): p. 3291-300.

76. Abramova, N.A., et al., *Interaction between replication protein A and p53 is disrupted after UV damage in a DNA repair-dependent manner.* Proc. Natl. Acad. Sci. USA, 1997. **94**(14): p. 7186-91.

77. Livengood, J.A., et al., *p53 Transcriptional activity is mediated through the SRC1-interacting domain of CBP/p300.* J. Biol. Chem., 2002. **277**(11): p. 9054-61.

78. Barlev, N.A., et al., *Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein.* J. Biol.Chem., 1995. **270**(33): p. 19337-44.

79. Levine, A.J., *p53, the cellular gatekeeper for growth and division.* Cell, 1997. **88**(3): p. 323-31.

80. Momand, J., et al., *The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation.* Cell, 1992. **69**(7): p. 1237-45.

81. Kubbutat, M.H., S.N. Jones, and K.H. Vousden, *Regulation of p53 stability by Mdm2.* Nature, 1997. **387**(6630): p. 299-303.

82. Huang, L.C., K.C. Clarkin, and G.M. Wahl, *Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest.* Proc. Natl. Acad. Sci. USA, 1996. **93**(10): p. 4827-32.

83. Linke, S., et al., *A reversible, p53-dependent G_0/G_1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage.* Genes & Dev., 1996. **10**: p. 934-947.

84. Graeber, T., et al., *Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors.* Nature, 1996. **379**: p. 88-91.

85. Milne, D., et al., *Phosphorylation of the p53 tumour-suppressor protein at three N-terminal sites by a novel casein kinase I- like enzyme.* Oncogene, 1992. **7**: p. 1361-1369.

86. Pise-Masison, C., et al., *Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotrophic virus type 1- transformed cells.* J. Virol., 1998. **8**: p. 6348-6355.

87. Shieh, S., et al., *DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2.* Cell, 1997. **3**: p. 325-334.

88. Lambert, P., et al., *Phosphorylation of p53 serine increases interaction with CBP.* J. Biol.Chem, 1998. **273**: p. 33048-33053.

89. Sakaguchi, K., et al., *Damage-mediated phosphorylation of human p53 threonine 18 through a cascade mediated by a casein 1-like kinase. Effect on Mdm2 binding.* J. Biol. Chem., 2000. **275**: p. 9278-9283.

90. Shieh, S., et al., *The human homologs of checkpoint kinases Chk1 and Cds1(Chk2) phosphorylate p53 at multiple DNA damage-inducible sites.* Genes & Dev., 2000. **3**: p. 289-300.

91. Dumaz, N., et al., *Critical roles for the serine 20, but not the serine 15, phosphorylation site and for the polyproline domain in regulating p53 turnover.* Biochem. J., 2001. **359**: p. 459-464.

92. Bulavin, D., et al., *Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation.* EMBO J., 1999. **23**: p. 6845-6854.

93. Buschmann, T., et al., *Jun NH_2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress.* Mol. Cell. Biol., 2001. **21**: p. 2743-2754.

94. Baudier, J., et al., *Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein.* Proc. Natl. Acad. Sci. USA, 1992. **89**: p. 11627-11631.

95. Appella, E. and C. Anderson, *Signaling to p53: breaking the post translational modification code.* Pathol. Biol., 2000. **48**: p. 227-245.

96. Ito, A., et al., *p300/CBP-mediated p53 acetylation is commonly induced by p53-activated agents and inhibited by MDM2.* EMBO J., 2001. **20**: p. 1331-1340.

97. Barlev, N.A., et al., *Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferase.* Mol.Cell, 2001. **8**: p. 1243-1254.

98. Gu, W. and R. Roeder, *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell, 1997. **4**: p. 595-606.

99. Sakaguchi, K., et al., *DNA damage activates p53 through a phosphorylation-acetylation cascade.* Genes & Dev., 1998. **18**: p. 2831-2841.

100. Liu, L., et al., *p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage.* Mol. Cell. Biol., 1999. **19**: p. 1202-1209.

101. el Deiry, W., *Regulation of p53 downstream genes.* Semin. Cancer Biol, 1998. **8**: p. 345-357.

102. Agarwal, M., et al., *The p53 network.* J.Biol. Chem., 1998. **273**: p. 1-4.

103. Miyashita, T. and J.C. Reed, *Tumor suppressor p53 is a direct transcriptional activator of the human bax gene.* Cell, 1995. **80**(2): p. 293-9.

104. Debbas, M. and E. White, *Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B.* Genes Dev, 1993. **7**(4): p. 546-54.

105. Howes, K.A., et al., *Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53.* Genes Dev, 1994. **8**(11): p. 1300-10.

106. Zhao, K., et al., *Crystal structure of the mouse p53 core DNA-binding domain at 2.7 A resolution.* J. Biol. Chem., 2001. **276**(15): p. 12120-7.

107. Clore, G.M., et al., *High-resolution structure of the oligomerization domain of p53 by multidimensional NMR.* Science, 1994. **265**(5170): p. 386-91.

CHAPTER 2

TFIIEα SHARES A COMMON BINDING SITE WITH THE AADs OF p53 AND VP16 ON THE hTFIIH SUBUNIT Tfb1

Introduction

In the cell, activators function by binding to specific DNA sequences and stimulating several stages of the transcription process through protein/protein interactions. One class of activators contacts numerous targets via their acidic activation domains (AADs). Known targets for AADs include the TATA-binding protein (TBP) of transcription factor IID (TFIID) [1], TBP-associated factors (TAFs) [2-4], transcription factor IIB (TFIIB) [5], and transcription factor IIH (TFIIH) [6]. The interaction between AADs and the p62 (human)/Tfb1 (yeast) subunit of TFIIH is particularly interesting. The ability of AADs to stimulate the transition from initiation to elongation is directly associated with their ability to interact with p62/Tfb1.

First discovered in mammalian cells, TFIIH has been identified in human (TFIIH), rat (factor δ), and yeast (factor b)[7-9]. Highly homologous in structure and function, yeast and human TFIIH consists of nine subunits grouped into two subcomplexes referred to as the core and CAK subcomplexes. The core subcomplex has six subunits XPB (Ssl2), p62 (Tfb1), p52 (Tfb2), p44 (Ssl1), p34 (Tfb4) and XPD (Rad3) whereas the CAK subcomplex, contains cdk7 (Kin28), cyclin H (Ccl1), and MAT1 (Tfb3) [10]. TFIIH is part of the preinitiation complex (PIC) and is recruited into the PIC by TFIIEα. Interestingly, TFIIH is the only general transcription factor possessing enzymatic activities including a DNA-dependent ATPase activity,

protein kinase activity, and an ATP-dependent DNA helicase activity. Currently, there are several functions attributed to the general transcription factor IIE (TFIIE). TFIIE exists as a heterotetramer *in vivo* composed of two subunits, TFIIEα and TFIIEβ [11]. TFIIEα is highly acidic with a pI of 4.5 and a molecular mass of 56KDa. TFIIEβ is a 34KDa basic protein with a pI of 9.5. Both of these subunits are necessary for TFIIE functions. In the step-wise assembly model, TFIIE enters the PIC after the association of RNAPII and transcription factor IIF (TFIIF) [11]. Following this step, TFIIE is responsible for recruiting TFIIH to the PIC [12]. TFIIEs remaining functions primarily involve regulating the enzymatic activities of TFIIH. Through various interactions with TFIIH, TFIIE is able to repress the helicase activity, stimulate CTD phosphorylation, and stimulate TFIIH-dependent ATP hydrolysis. In addition, it is believed that in cooperation with TFIIH, TFIIE aids in promoter clearance [13-15].

TFIIEα contains many acidic amino acid clusters and the extreme carboxyl-terminal domain (residues 336-439 in humans) is particularly acid-rich. Mutation within the acid-rich carboxyl-terminal domain of TFIIEα reduces basal transcription by 90% and this domain has been shown to interact directly with the p62 subunit of TFIIH [14]. Interestingly, this acid-rich domain shares sequence similarities with AADs.

In addition to p53, Herpes Simplex viral protein 16 (VP16) is another gene-specific transcriptional activator containing a bifunctional AAD. Upon lytic infection by the double stranded DNA herpes simplex virus (HSV), viral genes are expressed in three phases; immediate early (IE), delayed early (DE), and late (L). During infection, VP16 is responsible for initiating transcription of IE genes. Synthesized late in infection with 400-600 molecules per virion, VP16 is transcribed and replicated in the nucleus. Regulation of transcription during the IE phase is maintained by a multiprotein regulatory complex consisting of VP16, several other viral proteins,

and numerous host proteins. VP16 contains two distinct functional domains, a carboxyl-terminal AAD and an amino-terminal core domain required for protein/protein interactions. Although these domains do not depend on each other for function, both are required for transcriptional activation.

The unusually strong AAD of VP16 (residues 412-490), is responsible for activating transcription [16-18]. This domain can be further divided into two subdomains designated $VP16N_{(412-450)}$ and $VP16C_{(450-490)}$. Previous research has shown that each subdomain is able to independently activate transcription when fused to a heterologous DNA-binding domain [2]. Mutational analysis of the full AAD indicates that aromatic and hydrophobic residues within these two subdomains play an important role in activation. In particular, Leu^{439}, Phe^{442}, Leu^{444}, and Phe^{475} were identified as critical hydrophobic/aromatic residues [19]. VP16 has also been shown to interact with many components of the transcriptional machinery. Targets of VP16 include TFIIB, TFIID, TFIIH, TBP, TAFs as well as RNA Polymerase II [2, 5, 6, 20].

Like other AADs, it appears that the AAD of VP16 is disordered in the free state. Two-dimensional NMR studies of this domain showed an absence of any secondary structure [21]. In addition, fluorescence quenching, time-resolved intensity decay and time-resolved anisotrophy decay also revealed the absence of secondary structure elements [22]. As seen with other AADs, the AAD of VP16 adopts structure upon interaction with a target protein. For example, $VP16C_{(452-490)}$ forms an amphipathic α-helix following binding to $hTAF_{II}31$ [23]. $VP16C_{(452-490)}$ interacts with the amino-terminus of $hTAF_{II}31_{(1-181)}$ through hydrophobic interactions and salt-bridge contacts with key residues including Phe^{479}, Leu^{483}, as well as Asp^{472}. The pattern of hydrophobic residues found at the VP16/$hTAF_{II}31$ interface is similar to what has been observed with other AADs when bound to their targets.

The AADs of VP16 and p53 share similar sequences and functions. In addition, their amino acid composition is very similar to the amino acid composition of the acid-rich carboxyl-terminal domain of TFIIEα. Interestingly, previous research has shown that p53, VP16, and TFIIEα all directly interact with both the yeast and human Tfb1/p62 subunit of TFIIH. Given the similarities in their sequence, our goal was to investigate whether p53, TFIIEα, and VP16 share the same binding site on Tfb1/p62.

Materials and Methods

Cloning of recombinant proteins

The GST-$p62_{(1-127)}$ fragment was generated by taking $p62_{(1-548)}$ DNA in the pGEX-2T (Amersham) vector (gift from Dr. John Laadias, Harvard University) and creating a blunt end at amino acid 127. Both the pGEX-2T vector and the $p62_{(1-127)}$ insert were digested in a 100 µl total volume of BamHI reaction buffer (10X BamHI buffer, 80 units BamHI enzyme (NEB), 10X BSA) for 2 hours at 37°C. This resulted in the pGEX-2T vector and the $p62_{(1-127)}$ insert both having one BamHI end and one blunt end. To the $p62_{(1-127)}$ insert and pGEX-2T BamHI digests, 10X DNA loading dye was added and 60µl of each loaded into four wells of a 1.2% low melting agarose gel. The vector and $p62_{(1-127)}$ bands were cut from the gel and the DNA obtained using a gel extraction kit (Qiagen). Next, 2µl of the pGEX-2T vector DNA and 25µl of the $p62_{(1-127)}$ insert DNA were ligated for 2 hours at room temperature in 35 µl total volume ligation buffer (10X T4 DNA ligase buffer, 400 units T4 DNA ligase). The ligation was then transformed into 50 µl of Top10F' (Invitrogen) competent cells. Colonies were screened through expression of fusion proteins from isopropyl-D-thiogalactoside (IPTG) (Inalco) induced

bacterial cultures and verified by dideoxynucleotide sequencing. The GST-Tfb1$_{(1-115)}$ clone inserted into a pGEX-2T plasmid vector, was given by the laboratory of Dr. Jack Greenblatt (University of Toronto). The p53 deletion mutants used (GST-p53$_{(1-72,\ 1-40,\ 20-73)}$) were generously donated by Dr. Rong Li (University of Virginia). GST-VP16$_{(413-490)}$ was generously given by Dr. Steven Triezenburg (Michigan State University). The p53$_{(25-65)}$ peptide used in the 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) experiment was provided by Dr. Ettore Appella (National Institute of Health).

Expression and purification of Glutathione-S-Transferase (GST)-fusion proteins

(1) Tfb1$_{(1-115)}$. The GST-Tfb1$_{(1-115)}$ fragment used in the *in vitro* binding experiments was expressed from a pGEX-2T vector as a fusion protein in the *E. coli* host strain TOPP2 (Stratagene). The cells were grown at 37°C and protein expression was induced for 3 hours at 30°C with 0.7 mM IPTG. The cells were harvested by centrifugation and resuspended in EBC buffer (50 mM Tris-Cl pH 8.0, 120 mM NaCl, 0.5% NP-40, 2 mM DTT). The cells were then lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatant from the centrifugation was incubated for 1 hour with 3 mL of Glutathione Sepharose (GSH) resin (Amersham) at 4°C. Following incubation, the resin was collected by centrifugation and washed twice with NETN buffer (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.05% SDS, 1 mM DTT) and once in phosphate-buffered saline (PBS) pH 7.4 (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The fusion protein was eluted off the resin by rotating for ten minutes in Elution buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM DTT, 15 mM reduced Glutathione). The eluted fusion protein was dialyzed into

Freezing buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 20% glycerol, 2 mM DTT) and stored at -80°C.

(2) $p62_{(1-127)}$. The GST-$p62_{(1-127)}$ fragment used in the *in vitro* binding experiments was expressed from a pGEX-2T vector as a GST-fusion protein in an *E. coli* host strain Top10F'. The cells were grown at 37°C and protein expression was induced for 4 hours at 30°C with 0.7 mM IPTG. The cells were harvested by centrifugation and resuspended in EBC buffer. The cells were then lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatant from the centrifugation was incubated for 1 hour with 3 mL of GSH resin at 4°C. Following incubation, the resin was collected by centrifugation and washed twice with NETN buffer and once in PBS pH 7.4. The resin was diluted 1:1 with NETN buffer and stored at -80°C for future use in *in vitro* binding assays.

Protein expression and purification

(1) $p53_{(1-72)}$, $p53_{(1-40)}$, $p53_{(20-73)}$, TFIIE$\alpha_{(336-439)}$, and $VP16_{(413-490)}$. GST-p53 fragments$_{(1-72, 1-40, 20-73)}$ were expressed from the pGEX-2TK vector as a GST-fusion protein in *E. coli* host strain TOPP2. GST-$VP16_{(413-490)}$ and GST-TFIIE$\alpha_{(336-439)}$ were expressed in the pGEX-2T vector in TOPP2 and BL21 (DE3) pLysS (Novagen) cell lines, respectively. The cells were grown overnight at 37°C, and protein expression was induced for 4 hours with 0.7 mM IPTG at 30°C. The cells were harvested by centrifugation, resuspended in GST lysis buffer (20 mM Tris-HCl pH 7.4, 1mM EDTA, 1M NaCl, 1mM DTT), lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatants were incubated for 1 hour with 10 mL of GSH resin at 4°C. Following incubation, the resins were washed with lysis buffer three times followed by equilibration with PBS pH 7.4 containing 1 mM DTT. The resins containing the

bound fusion proteins were then incubated overnight at room temperature with 100 units of thrombin (Calbiochem) to cut the $p53_{(1-72)}$, $p53_{(1-40)}$, $p53_{(20-73)}$, $TFIIE\alpha_{(336-439)}$, and $VP16_{(413-490)}$ fragments from the GST. Following thrombin cleavage, the supernatants were incubated for 1 hour with Benzamidine Sepharose (Amersham), filtered to remove the Benzamidine Sepharose, dialyzed into buffer A (20 mM phosphate buffer pH 6.5, 1 mM DTT, 1 mM EDTA), and then applied to a Q-Sepharose High Performance (Amersham) column (75 mL) equilibrated with buffer A. The $p53_{(1-72)}$, $p53_{(1-40)}$, $p53_{(20-73)}$, $TFIIE\alpha_{(336-439)}$, and $VP16_{(413-490)}$ fragments were eluted from the column using a gradient (from 0 to 100% over 700 mL) of buffer B (20 mM phosphate buffer, 1 mM DTT, 1 mM EDTA, and 1 M NaCl). Fractions containing the purified $p53_{(1-72)}$, $p53_{(1-40)}$, $p53_{(20-73)}$, $TFIIE\alpha_{(336-439)}$, and $VP16_{(413-490)}$ fragments were then pooled, dialyzed overnight into buffer A, and concentrated for NMR spectroscopy.

(2) $Tfb1_{(1-115)}$. GST-$Tfb1_{(1-115)}$ was expressed from the pGEX-2T vector as a fusion protein in the *E. coli* host strain TOPP2. The cells were grown overnight at 37°C, and protein expression was induced for 4 hours with 0.7 mM IPTG at 30°C. The cells were harvested by centrifugation, resuspended in GST lysis buffer, lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatants were incubated for 1 hour with 10 mL of GSH resin at 4°C. Following incubation, the resins were washed with GST lysis buffer three times followed by equilibration with PBS pH 7.4 containing 1 mM DTT. The resins containing the bound fusion proteins were then incubated overnight at room temperature with 100 units of thrombin to cut $Tfb1_{(1-115)}$ from the GST. Following thrombin cleavage, the supernatants were incubated for 1 hour with Benzamidine Sepharose, filtered to remove Benzamidine Sepharose, dialyzed into buffer A, and then applied to a S-Sepharose High Performance (Amersham) column (75 mL) equilibrated with buffer A. The $Tfb1_{(1-115)}$ fragment was eluted from the

column using a gradient (from 0 to 100% over 700 mL) of buffer B. Fractions containing the purified $Tfb1_{(1-115)}$ fragment were then pooled, dialyzed overnight into buffer A, and concentrated for NMR spectroscopy.

(3) $Tfb1_{(1-115)}$, $p53_{(20-73)}$ and $TFIIE\alpha_{(336-439)}$ for NMR Spectroscopy. $Tfb1_{(1-115)}$ was expressed from the pGEX-2T vector as a GST-fusion protein in *E.coli* host strain TOPP2. GST-$p53_{(20-73)}$ was expressed in the pGEX-2TK vector in the Rosetta (Novagen) cell line. GST-$TFIIE\alpha_{(336-439)}$ was expressed in the pGEX-2T vector in the BL21 (DE3) pLysS (Novagen) cell line. Uniform (>98%) ^{15}N labeling was performed by growing the cells in a modified minimal medium containing $^{15}NH_4Cl$ as sole nitrogen sources. The purification was carried out as described for the unlabeled $p53_{(20-73)}$, $TFIIE\alpha_{(336-439)}$ and $Tfb1_{(1-115)}$ fragments.

In Vitro Binding Assays

All *in vitro* binding experiments were performed using 12.5 µl of GSH resin in 0.5X Superdex buffer (25 mM Hepes, pH 7.9, 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 150 mM KCl , 20% glycerol, 0.1% Nonidet P-40, 1 mM EDTA). For each assay, 1 µM of Glutathione-S-Transferase (GST), or GST-$Tfb1_{(1-115)}$ fusion protein was incubated with the GSH resin for 1 hour at 4°C with constant rotation. The 1 µM GST-$p62_{(1-127)}$ was not eluted therefore, we did not need to rebind it to the resin. The resins were then washed two times with 0.5 mL of 0.5X Superdex buffer. Purified p53 fragments were then added at various concentrations (0.1 µM-1 µM) to the washed resin in 0.5 mL of 0.5X Superdex buffer and incubated for 1 hour at 4°C with constant rotation. The resin was then washed twice as before with 0.5 mL of 0.5X Superdex buffer. Following the wash step, 25 µl of Elution Buffer (100 mM Tris-HCl pH 6.8, 140 mM SDS, 200 mM DTT, 2.3% Bromophenol Blue, 20% glycerol) was added to elute the bound protein. The

samples were heated for 10 minutes at 60°C in elution buffer and spun down in a centrifuge to pellet the resin. The supernatant (20 μl) was loaded onto a 12% acrylamide gel and separated by electrophoresis using a Tris-Glycine buffer (10% Tris-Glycine[1.92 M Glycine, 0.25 M Tris], 10% SDS). The proteins were then transferred to an Immobilon-P membrane (Millipore) at 4°C in 1X Tris-Glycine for 1 hour at 44V. The membrane was incubated overnight in 50 mL of 1X TBS/Blotto (100 mM Tris-Cl pH 8.0, 1.5 M NaCl, 5% nonfat dry milk) with constant shaking. The following day the membrane was incubated in 10 mL of TBST/Blotto (100 mM Tris-Cl pH 8.0, 1.5 M NaCl, 0.05% Tween 20, 5% nonfat dry milk) and a 1:10000 dilution of an anti-p53 primary antibody (p53 DO1 [epitope 11-35] or Pab 1801[epitope 32-79]; Santa Cruz) for 1 hour. Excess primary antibody was removed by three successive 5-minute washes with TBST. The washed membrane was then incubated in 10 mL of TBST/Blotto and a 1:5000 dilution of secondary antibody (goat-antimouse IgG-HRP sc-2055; Santa Cruz) for 30 minutes. The membrane was washed four times with TBST for five minutes to remove the excess secondary antibody. The protein was detected by autoradiography through chemiluminescence using ECL Plus (Amersham). The membrane was Coomassie stained to ensure equal molar concentrations of protein inputs for the GST control and the GST-fusion proteins.

Results

Tfb1$_{(1-115)}$ interacts with the acid-rich CTD of TFIIEα$_{(336-439)}$

In eukaryotic transcription of mRNA, one of the major roles of TFIIE is to recruit TFIIH to the PIC [12]. In particular, the acid-rich carboxyl-terminal domain of the α-subunit of TFIIE is required for interaction with TFIIH both *in vivo* and *in vitro* [14, 24]. The amino acid

composition of the carboxy-terminal domain of TFIIEα (TFIIEα$_{(336-439)}$) is very similar to the AADs of activator proteins. Using *in vitro* binding studies, it has been shown that the amino-terminus of the yeast TFIIH subunit Tfb1$_{(1-115)}$ binds the carboxy-terminal domain of TFIIEα$_{(336-439)}$ [Greenblatt and Kobor (personal communication)]. We verified this by forming a complex between Tfb1$_{(1-115)}$ and TFIIE$_{(336-439)}$ and analyzing it using 2D NMR experiments. The 1:1 complex was prepared by titrating a solution of 1.2 mM TFIIEα$_{(336-439)}$ into a solution containing 1.2 mM ^{15}N-labeled Tfb1$_{(1-115)}$. Following concentration, the resulting complex was analyzed using a 2D ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) experiment and compared to a 2D ^{1}H-^{15}N HSQC of free ^{15}N-labeled Tfb1$_{(1-115)}$. An overlay of the ^{1}H-^{15}N HSQC spectra of free ^{15}N-labeled Tfb1$_{(1-115)}$ (black) and of the ^{15}N-labeled Tfb1$_{(1-115)}$ / TFIIEα$_{(336-439)}$ complex (red) showed considerable changes (Δ (^{1}H) $\geq$ 0.05 ppm and/or Δ (^{15}N) $\geq$ 0.5 ppm) in both ^{1}H and ^{15}N chemical shifts indicating as predicted that we had formed the complex (Figure 2.1A). Calculated differences in both ^{1}H and ^{15}N chemical shifts of Tfb1$_{(1-115)}$ between the free and the TFIIEα complexed state revealed that 35 out of 115 amide signals of Tfb1$_{(1-115)}$ undergo significant changes in chemical shift. This information was used to map the binding site for TFIIEα$_{(336-439)}$ on the sequence of Tfb1$_{(1-115)}$ (Figure 2.1B). Of the eight β-strands (β1-β8) and one α-helix (α1) in Tfb1$_{(1-115)}$, the α-helix, β6, and β8 seem to play an integral role in the interaction, and in particular α1 as 13 of the 35 shifted residues are located in and around this secondary structure element.

The AAD of p53 binds the amino-terminus of the Tfb1 subunit of yeast TFIIH and its human homologue p62

Once it was determined that the acid-rich carboxyl-terminal domain of TFIIEα$_{(336-439)}$ binds the amino-terminus of Tfb1, we wanted to examine whether other acid-rich domains with

similar sequence would interact in the same manner. Previous research has shown that the AAD of p53 interacts with the Tfb1 subunit of yeast TFIIH and its human homologue p62 [6]. In order to evaluate the interaction between p53 and Tfb1/p62, we first purified fragments of the p53 $AAD_{(1\text{-}72,1\text{-}40,20\text{-}73)}$, GST-$Tfb1_{(1\text{-}115)}$ and GST-$p62_{(1\text{-}127)}$ (Figure 2.2). First, both GST-fusion proteins (GST-$Tfb1_{(1\text{-}115)}$ and GST-$p62_{(1\text{-}127)}$) were tested for their ability to pull down the full length AAD of p53 ($p53_{(1\text{-}72)}$). $p53_{(1\text{-}72)}$ exhibited binding to both GST-$p62_{(1\text{-}127)}$ and GST-$Tfb1_{(1\text{-}115)}$ (Figure 2.3). Based on these results, the full length AAD of p53 can interact specifically with either the amino-terminus of p62 or Tfb1.

Next, we attempted to define the role of each subdomain within the p53 AAD for interaction with $Tfb1_{(1\text{-}115)}$ and $p62_{(1\text{-}127)}$. The first subdomain (AAD1: $p53_{(1\text{-}40)}$) is essential for p53 interaction with MDM2, $TAF_{II}40$, $TAF_{II}31$, and the KIX domain of the CREB binding protein (CBP) [4, 23, 25, 26]. Within AAD1, residues 13-23 are highly conserved in a number of species and mutations within these 11 amino acids inhibits binding of p53 to several of the above mentioned targets. As with $p53_{(1\text{-}72)}$, purified recombinant $p53_{(1\text{-}40)}$ was incubated with both GST-$Tfb1_{(1\text{-}115)}$ and GST-$p62_{(1\text{-}127)}$ to determine if this region was sufficient for binding (Figure 2.4). These experiments demonstrated that the $p53_{(1\text{-}40)}$ fragment was not capable of binding to either $Tfb1_{(1\text{-}115)}$ or $p62_{(1\text{-}127)}$ under conditions where binding was observed with $p53_{(1\text{-}72)}$. Based on these results, it appears that either AAD1 is not required or insufficient for binding, suggesting perhaps AAD2 is mandatory for binding to $Tfb1_{(1\text{-}115)}$ or $p62_{(1\text{-}127)}$. To evaluate whether AAD2 was sufficient for binding $Tfb1_{(1\text{-}115)}$, the *in vitro* binding assay was repeated with $p53_{(20\text{-}73)}$. We observed that $p53_{(20\text{-}73)}$ binds to $Tfb1_{(1\text{-}115)}$ in a manner very similar to $p53_{(1\text{-}72)}$ and this suggests that the p53 binding site for Tfb1/p62 is located almost entirely within AAD2 and is different then the domain required for MDM2 binding (Figure 2.5).

Tfb1$_{(1-115)}$ interacts with both TFIIE$\alpha_{(336-439)}$ and p53$_{(25-65)}$ through a similar binding site

To further determine the minimal region of p53 required for Tfb1$_{(1-115)}$ binding, a peptide completely lacking the critical hydrophobic residues present in AAD1 was chemically synthesized (p53$_{(25-65)}$). This peptide was chosen because it lacks the additional hydrophobic residue critical for MDM2 interaction (Trp^{23}) present in p53$_{(20-73)}$. In order to determine if p53$_{(25-65)}$ was sufficient for binding to Tfb1$_{(1-115)}$, and to map the binding surface of p53$_{(25-65)}$ on Tfb1$_{(1-115)}$, a 2D ^{1}H-^{15}N HSQC experiment was performed with ^{15}N-labeled Tfb1$_{(1-115)}$ and unlabeled p53$_{(25-65)}$. As with TFIIE$\alpha_{(336-439)}$, a solution of 1.2 mM unlabeled p53$_{(25-65)}$ was titrated into a sample containing 1.2 mM ^{15}N-labeled Tfb1$_{(1-115)}$. The NMR experiments demonstrated that p53$_{(25-65)}$ was capable of binding to Tfb1$_{(1-115)}$ since there was a significant change in the chemical shift of several residues (Δ (^{1}H) $\geq$ 0.05 ppm and/or Δ (^{15}N) $\geq$ 0.5 ppm) in both the ^{1}H and ^{15}N chemical shifts indicating complex formation (Figure 2.6A). Based upon this spectrum, we define the region of p53 required for interaction with Tfb1$_{(1-115)}$ as amino acids 25 to 65. Therefore, p53 interacts with Tfb1$_{(1-115)}$ through a region of p53 that is distinct from the region required for interaction with MDM2. An overlay of the free ^{15}N-labeled Tfb1$_{(1-115)}$ and ^{15}N-labeled Tfb1$_{(1-115)}$ / p53$_{(25-65)}$ shows a shift in 52 of the 115 Tfb1$_{(1-115)}$ residues. Interestingly, 33 of the 35 residues shifted in the ^{15}N-labeled Tfb1$_{(1-115)}$ / TFIIE$\alpha_{(336-439)}$ complex are also shifted in the ^{15}N-labeled Tfb1$_{(1-1195}$ / p53$_{(25-65)}$ complex. Therefore, it appears that TFIIEα and p53 interact with Tfb1 through the same binding region and could potentially compete for the binding site in the cell.

The AAD of VP16 binds the amino terminus of Tfb1

The carboxyl-terminal AAD of VP16 has been shown to directly target both Tfb1 and p62 [6]. This interaction has been mapped between amino acids 413 and 490 of VP16 [6]. To analyze the interaction between $Tfb1_{(1-115)}$ and $VP16_{(413-490)}$ we performed 2D ^{1}H-^{15}N HSQC experiments. The 1:1 complex was prepared by titrating a solution of 1.2 mM unlabeled $VP16_{(413-490)}$ into a solution containing 1.2 mM ^{15}N-labeled $Tfb1_{(1-115)}$. An overlay of the ^{1}H-^{15}N HSQC spectra of free ^{15}N-labeled $Tfb1_{(1-115)}$ (black) and of the ^{15}N-labeled $Tfb1_{(1-115)}$ / $VP16_{(413-490)}$ complex (red) showed considerable changes (Δ (^{1}H) $\geq$ 0.05 ppm and/or Δ (^{15}N) $\geq$ 0.5 ppm) in both ^{1}H and ^{15}N chemical shifts indicating as predicted that we had formed the complex (Figure 2.7A). Calculated differences in both ^{1}H and ^{15}N chemical shifts of $Tfb1_{(1-115)}$ between the free and the VP16 complexed state revealed that 55 out of 115 amide signals of Tfb1 undergo significant changes in chemical shift. This information was used to map the binding site for $VP16_{(413-490)}$ on the sequence of $Tfb1_{(1-115)}$ (Figure 2.7B). As seen with the TFIIEα/Tfb1 and p53/Tfb1 complexes, α1, β6, and β8 seem to play an integral role in the interaction, and α1 is particularly important as 17 of the 55 shifted residues are located in and around this secondary structure element.

Discussion

The interplay of activator proteins and transcriptional machinery has an important role in regulating the rate of eukaryotic transcription. In the step-wise assembly model of the PIC, activators stimulate the recruitment of several transcription factors including TFIIE to the promoter [27]. Since one of the major functions of TFIIE is the recruitment of TFIIH, it is interesting to note that the acid-rich carboxy-terminus of its α-subunit is similar in amino acid

composition to activator proteins, containing AADs. In this study, we have shown using a combination of *in vitro* binding studies and NMR spectroscopy that the acid-rich domains of TFIIEα, p53, and VP16 interact with $Tfb1_{(1-115)}$. Interestingly, the interaction of each of these targets with $Tfb1_{(1-115)}$ affect several residues in and around its α1 helix. This helix contains a LQQII sequence similar to the LXXLL and LXXI/HIXXXL/I motifs found in nuclear hormone receptor (NR) coactivators and NR corepressor proteins, respectively [28, 29]. Interaction with the α1 helix, in particular the LQQII sequence, was first observed in our $Tfb1_{(1-115)}$ / $TFIIE\alpha_{(336-439)}$ data. Subsequent studies with the $Tfb1_{(1-115)}$ / $p53_{(25-65)}$ and $Tfb1_{(1-115)}$ / $VP16_{(412-490)}$ complexes revealed that residues undergoing significant chemical shift changes in these complexes were strikingly similar to those seen in $Tfb1_{(1-115)}$ / $TFIIE\alpha_{(336-439}$. Based on these results, it appears that $TFIIE_{(336-439)}$, $p53_{(25-65)}$, and $VP16_{(412-490)}$ may compete for the same binding site on $Tfb1_{(1-115)}$ *in vivo*.

Previous binding studies have demonstrated that TFIIEα (residues 336-439) interacts with $Tfb1_{(1-115)}$ *in vitro* (Greenblatt and Kobor, unpublished results). We have confirmed these results using NMR spectroscopy (Figure 2.1A). Residues of $Tfb1_{(1-115)}$ affected by complex formation with $TFIIE\alpha_{(336-439)}$ (Figure 2.1B, red), were interspersed throughout the domain, but the majority are found in the α1 helix. $TFIIE\alpha_{(336-439)}$ contains many acidic and hydrophobic residues that are possibly critical for this interaction.

The acid-rich domain of TFIIEα resembles AADs found in many transcriptional activators and we believe there is an important interplay between TFIIEα and AADs in regulating transcriptional processes. The AAD of p53 is contained within the first 72 amino acids of the protein [30]. To better define the region of interaction between $Tfb1_{(1-115)}$ and p53, constructs of the AAD evaluated included AAD1 ($p53_{(1-40)}$), AAD2 with a small portion of

AAD1 ($p53_{(20-73)}$), and AAD2 ($p53_{(25-65)}$). In our NMR and binding studies, the minimal region of p53 required for interaction with $Tfb1_{(1-115)}$ and $p62_{(1-127)}$ was located between amino acids 25 and 65 in the AAD2 region. This region is distinct from the region identified as the MDM2 binding site in p53 [31]. NMR studies of $Tfb1_{(1-115)}$ in the presence of $p53_{(25-65)}$ revealed interactions with various secondary structure elements present in the domain, but 15 of the 52 shifted residues are in the α1-helix. This correlates with data shown for the interaction of $TFIIE\alpha_{(336-439)}$ and $Tfb1_{(1-115)}$ and suggests the AAD of p53 may share a binding site with the acid-rich carboxyl-terminal domain of TFIIEα.

In addition, we examined interactions between the viral activator VP16 with $Tfb1_{(1-115)}$. The transactivation domain of VP16 encompasses 78 amino acids at its carboxy-terminus. The ability of the AAD of VP16 to bind $Tfb1_{(1-115)}$ was analyzed by NMR spectroscopy (Figure 2.7A). These studies confirmed complex formation and we again mapped residues of $Tfb1_{(1-115)}$ that experienced significant changes in chemical shift (Figure 2.7B, red). Interestingly, similar to what was seen with $TFIIE\alpha_{(336-439)}$ and $p53_{(25-65)}$, $VP16_{(413-490)}$ affected all residues in the α1 helix of $Tfb1_{(1-115)}$. Based on these data, the α1 helix of $Tfb1_{(1-115)}$ seems to play a prominent role in the interaction with each of these three targets.

The chemical shift changes in $Tfb1_{(1-115)}$ induced by binding to either $TFIIE\alpha_{(336-439)}$, $p53_{(20-73)}$, and $VP16_{(413-490)}$ are very similar. The α1 helix of $Tfb1_{(1-115)}$ (amino acids 97-110) seems to play an important role because 71% of residues in this region are shifted in each complex. As previously stated, the LQQII sequence present in the α1 helix is very similar to the LXXLL and LXXI/HIXXXL/I motifs seen in NR coactivators and repressors. These transcription factors interact with their respective targets via this highly conserved LXXLL motif which serves as the binding interface [32]. For example, estrogen receptor β uses an LXXLL

like motif to bind the corepressors N-CoR and SMRT and mutations in this sequence disrupts the interaction [33]. In addition, the α1 helix is composed of several hydrophobic residues which in other complexes of activators and their targets have been shown to be required for interaction [31, 34, 35]. Based on this preliminary data, the α1 helix of $Tfb1_{(1-115)}$, could feasibly be required for interaction with $TFIIE\alpha_{(336-439)}$, $p53_{(20-73)}$, or $VP16_{(413-490)}$.

Another interesting aspect of the complexes in this study is that each of the components is involved in the regulation of transcription. It has been shown that both p53 and VP16 stimulate initiation and elongation [36]. The ability of these activators to stimulate these stages of transcription correlates with their ability to interact with the p62/Tfb1 subunit of TFIIH. Because the acid-rich carboxyl-terminal domain of TFIIEα shares a common binding site with the AAD of p53 and VP16 on $Tfb1_{(1-115)}$, they may bind using a similar mechanism in order to stimulate elongation. Further structural and functional studies will lend insight to how these factors regulate transcription.

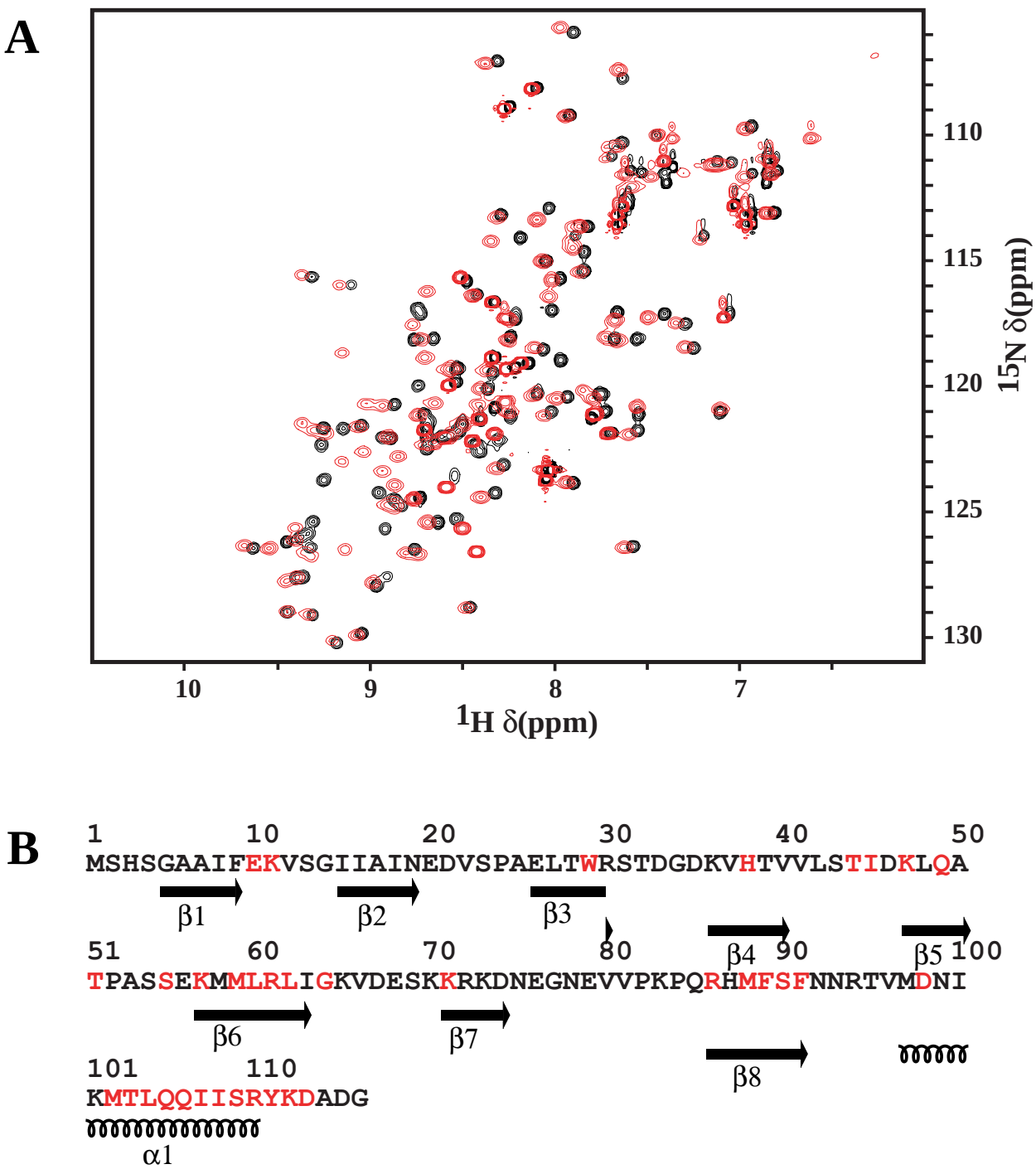


Figure 2.1. (A) Overlay of the 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Tfb1$_{(1\text{-}115)}$ in the absence (black) and presence (red) of unlabeled TFIIE$\alpha_{(336\text{-}439)}$. **(B)** Primary amino acid sequence of Tfb1$_{(1\text{-}115)}$ with residues showing significant chemical shift changes indicating complex formation with TFIIE$\alpha_{(336\text{-}439)}$ mapped onto the sequence (red).

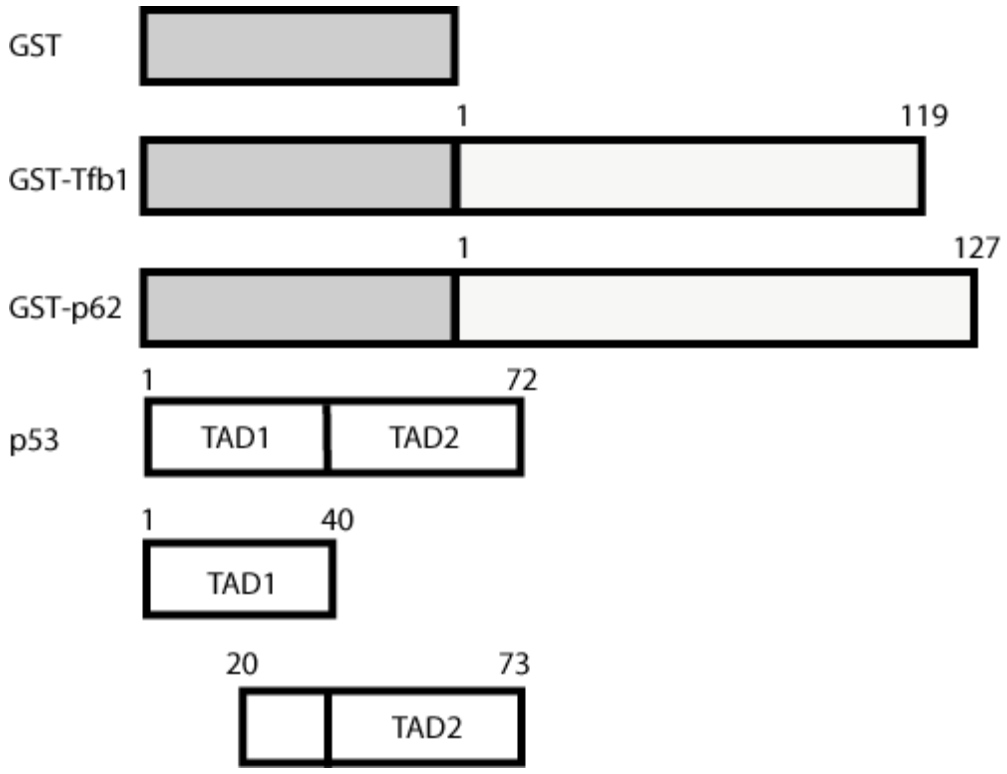


Figure 2.2. Schematic representation of proteins used in *in vitro* binding assays. In GST pull-down assays $Tfb1_{(1-115)}$ and $p62_{(1-127)}$ were used as GST-fusion proteins while $p53_{(1-72,1-40,20-73)}$ was purified protein.

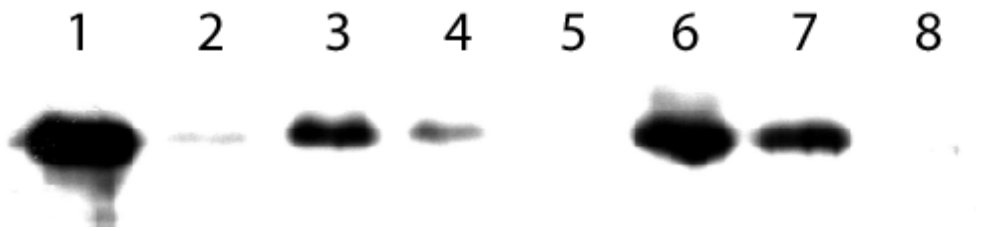


Figure 2.3. *In vitro* binding studies of $p53_{(1-72)}$ to the amino-terminus of p62 and Tfb1. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ were incubated with either 1 μM GST-$p62_{(1-127)}$ (lanes 3-5) or GST-$Tfb1_{(1-115)}$ (lanes 6-8). In the GST lane, 1 μM of purified $p53_{(1-72)}$ was incubated with 1 μM GST as a control (lane 2). The input lane is 0.5% $p53_{(1-72)}$ (lane 1).

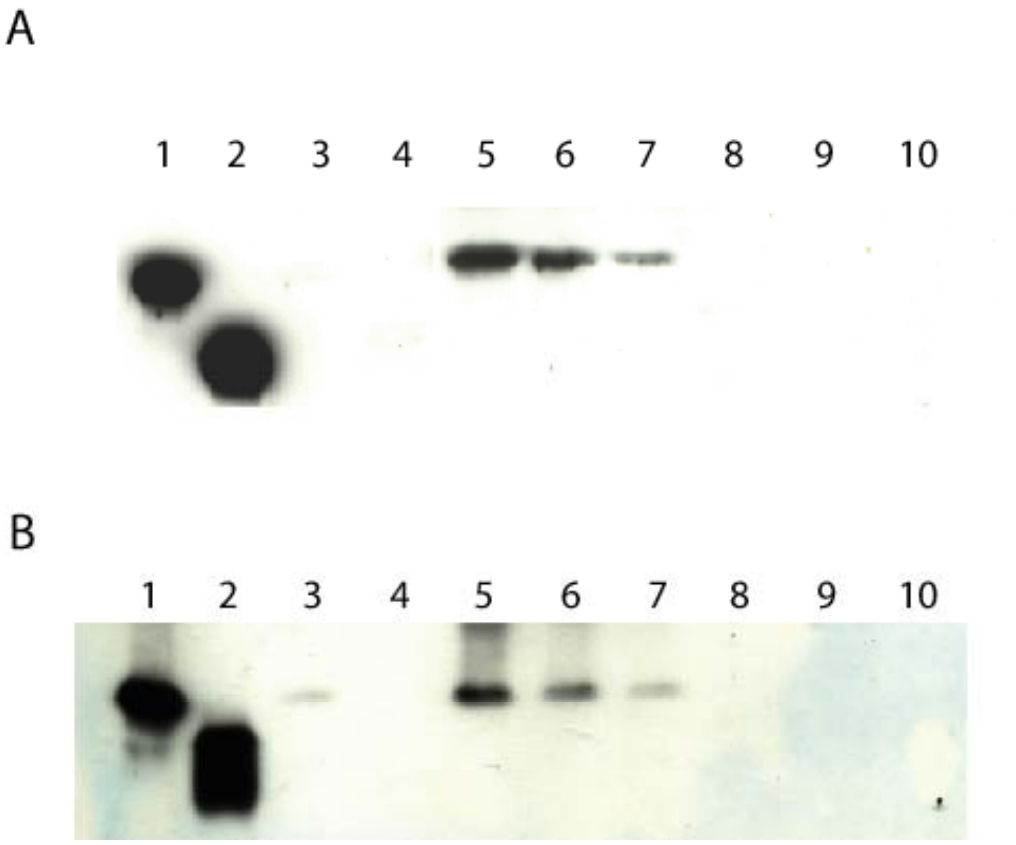


Figure 2.4. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(1-40)}$ to the amino-terminus of p62 and Tfb1. (A) Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-40)}$ (lanes 8-10) were incubated with 1 μM GST-$Tfb1_{(1-115)}$ **(B)** Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-40)}$ (lanes 8-10) were incubated with 1 μM GST-$p62_{(1-127)}$. In both experiments 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(1-40)}$ (lane 4) was incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(1-40)}$ (lane 2).

Figure 2.5. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(20-73)}$ to the amino-terminus of Tfb1. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(20-73)}$ (lanes 8-10) were incubated with 1 μM GST-$Tfb1_{(1-115)}$. In the GST lanes 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(20-73)}$ (lane 4) was incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(20-73)}$ (lane 2).

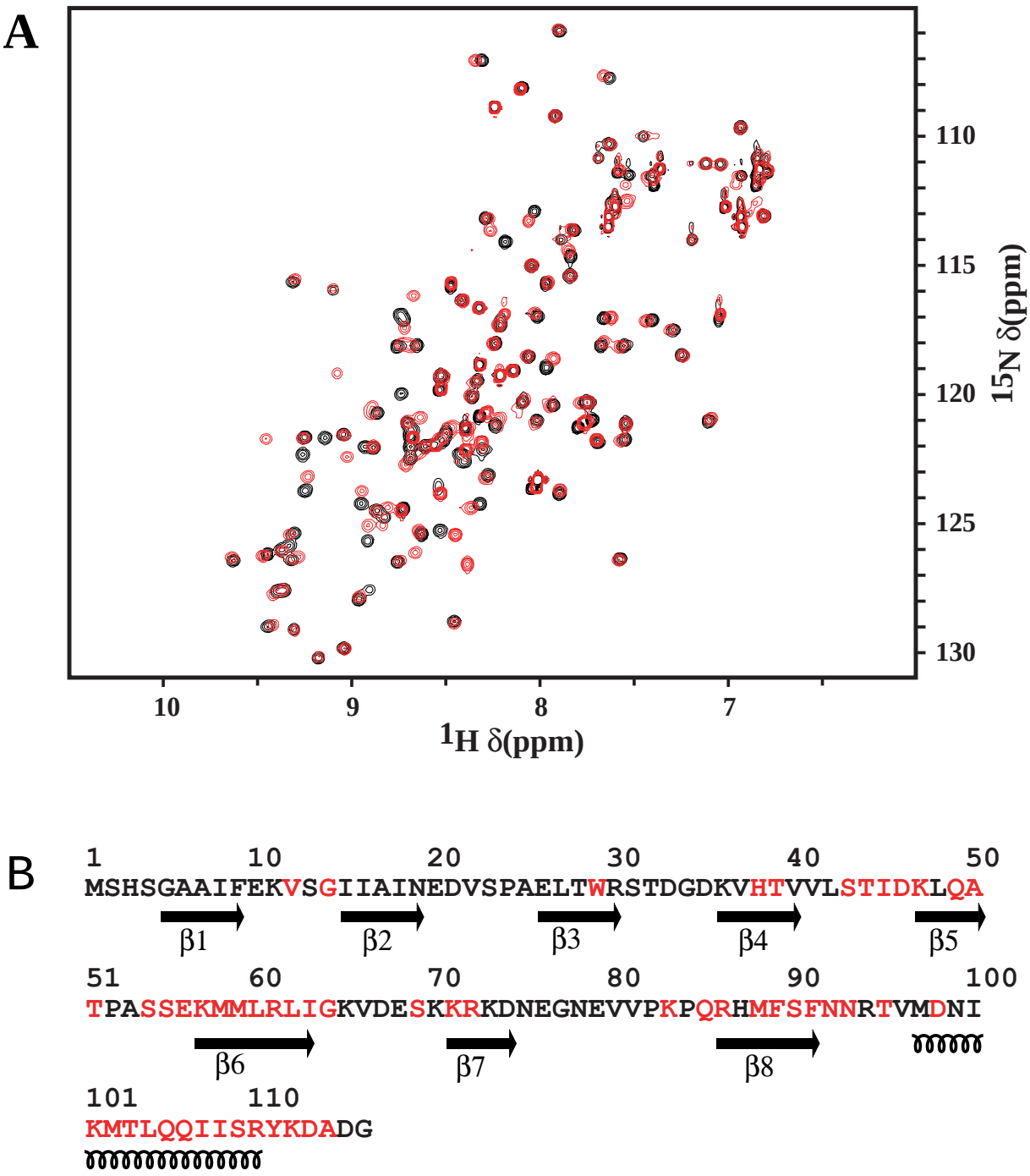
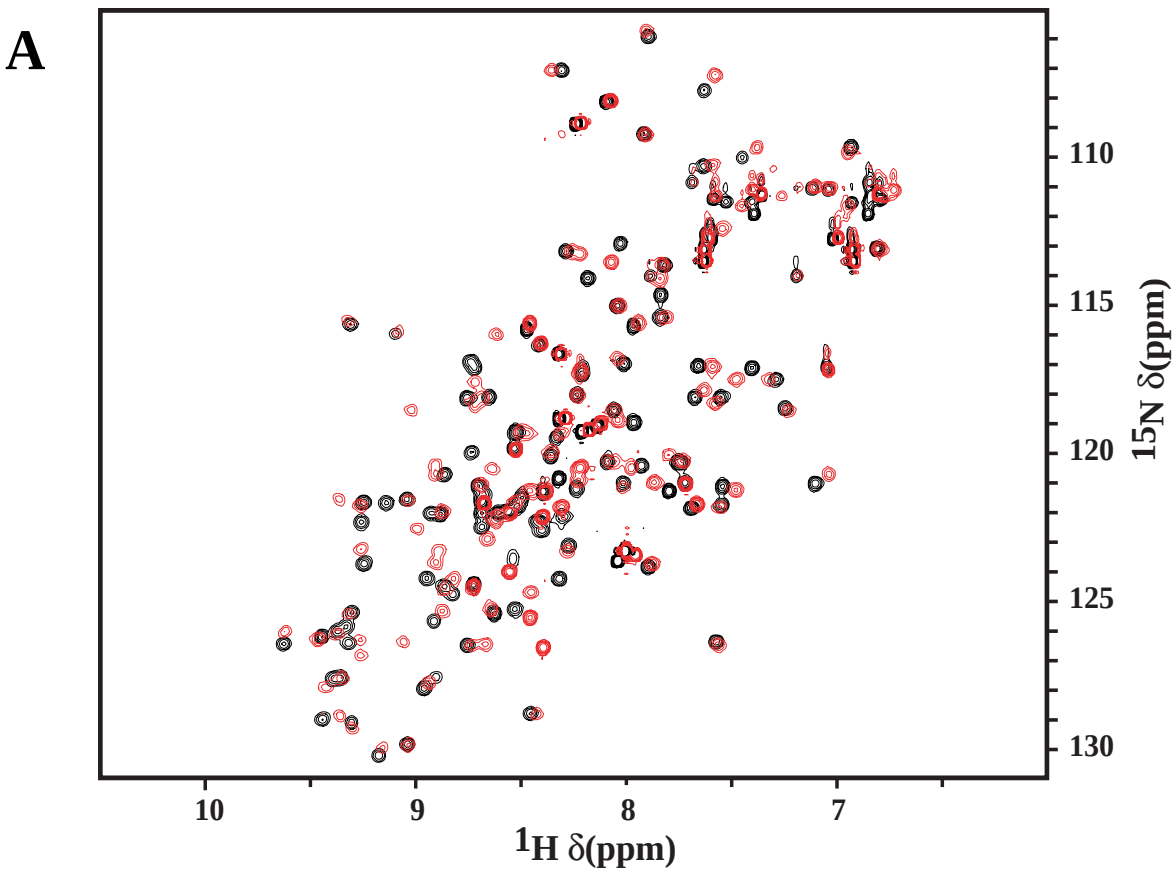


Figure 2.6. (A) Overlay of the 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Tfb1$_{(1-115)}$ in the absence (black) and presence (red) of unlabeled p53$_{(25-65)}$ **(B)** Primary amino acid sequence of Tfb1$_{(1-115)}$ with residues showing significant chemical shift changes indicating complex formation with p53$_{(25-65)}$ mapped onto the sequence (red).



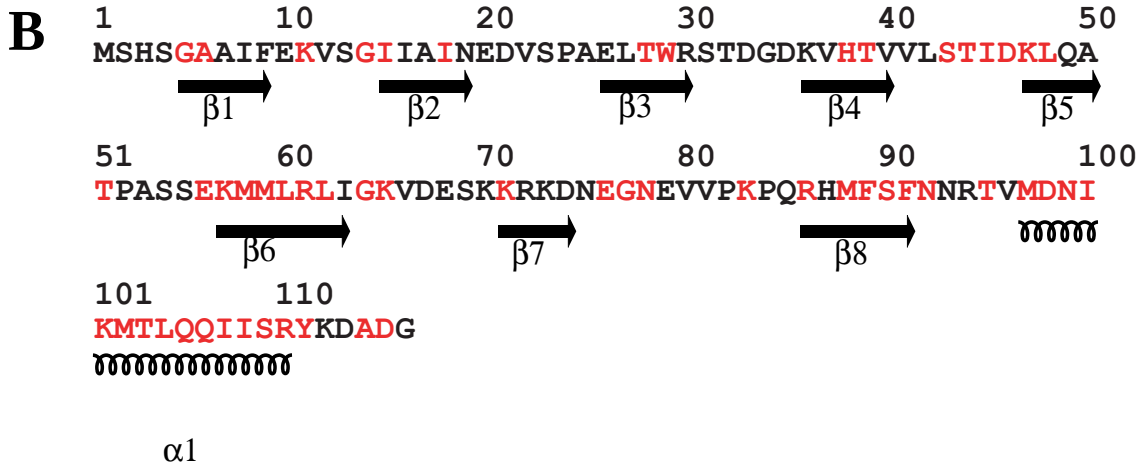


Figure 2.7. (A) Overlay of the 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Tfb1$_{(1\text{-}115)}$ in the absence (black) and presence (red) of unlabeled VP16$_{(413\text{-}490)}$. **(B)** Primary amino acid sequence of Tfb1$_{(1\text{-}115)}$ with residues showing significant chemical shift changes indicating complex formation with VP16$_{(413\text{-}490)}$ mapped onto the sequence (red).

References

1. Stringer, K., C. Ingles, and J. Greenblatt, *Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID.* Nature, 1990. **345**: p. 783-786.

2. Goodrich, J., et al., *Drosophila $TAF_{II}40$ interacts with both a VP16 activation domain and basal transcription factor TFIIB.* Cell, 1993. **75**: p. 519-530.

3. Gill, G., et al., *A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the $dTAF_{II}IIO$ component of the Drosophilia TFIID complex and mediates transcriptional activation.* Proc. Natl. Acad. Sci., 1994. **91**: p. 192-196.

4. Thut, C., et al., *p53 transcriptional activation mediated by coactivators $TAF_{II}40$ and $TAF_{II}60$.* Science, 1995. **267**: p. 100-104.

5. Lin, Y., et al., *Binding of general transcription factor TFIIB to an acidic activating region.* Nature, 1991. **353**: p. 569-571.

6. Xiao, H., et al., *Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53.* Mol. Cell. Biol., 1994. **14**: p. 7013-7024.

7. Flores, O., H. Lu, and D. Reinberg, *Factors involved in specific transcription by mammalian RNA polymerase II. Identification and characterization of factor IIH.* J. Biol. Chem., 1992. **267**(4): p. 2786-93.

8. Conaway, R.C. and J.W. Conaway, *An RNA polymerase II transcription factor has an associated DNA-dependent ATPase (dATPase) activity strongly stimulated by the TATA region of promoters.* Proc. Natl. Acad. Sci. U S A, 1989. **86**(19): p. 7356-60.

9. Feaver, W.J., et al., *CTD kinase associated with yeast RNA polymerase II initiation factor b.* Cell, 1991. **67**(6): p. 1223-30.

10. Frit, P., E. Bergmann, and J.M. Egly, *Transcription factor IIH: a key player in the cellular response to DNA damage.* Biochimie, 1999. **81**(1-2): p. 27-38.

11. Inostroza, J., O. Flores, and D. Reinberg, *Factors involved in specific transcription by mammalian RNA polymerase II. Purification and functional analysis of general transcription factor IIE.* J. Biol. Chem., 1991. **266**(14): p. 9304-8.

12. Buratowski, S., et al., *Five intermediate complexes in transcription initiation by RNA polymerase II.* Cell, 1989. **56**(4): p. 549-61.

13. Drapkin, R., et al., *Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II.* Nature, 1994. **368**(6473): p. 769-72.

14. Ohkuma, Y., et al., *Analysis of the role of TFIIE in basal transcription and TFIIH-mediated carboxy-terminal domain phosphorylation through structure-function studies of TFIIE-alpha.* Mol. Cell. Biol., 1995. **15**(9): p. 4856-66.

15. Maxon, M.E., J.A. Goodrich, and R. Tjian, *Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance.* Genes Dev., 1994. **8**(5): p. 515-24.

16. Sadowski, I., et al., *GAL4-VP16 is an unusually potent transcriptional activator.* Nature, 1988. **335**(6190): p. 563-4.

17. Triezenberg, S., R. Kingsbury, and S. McKnight, *Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early expression.* Genes Dev., 1988. **2**: p. 718-729.

18. Liu, Y., et al., *Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16.* Genes Dev., 1999. **13**(13): p. 1692-703.

19. Reiger, J., F. Shen, and S. Triezenberg, *Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator.* Proc. Natl. Acad. Sci., 1993. **90**: p. 883-887.

20. Ingles, C., et al., *Reduced binding of TFIID to transcriptionally compromised mutants of VP16.* Nature, 1991. **351**: p. 588-590.

21. O'Hare, P. and S. Williams, *Structural studies of the acidic transactivation domain of the Vmw65 protein of herpes simplex virus using ^{1}H NMR.* Biochemistry, 1992. **31**: p. 4150-4156.

22. Shen, F., et al., *Transcriptional activation domain of the herpesvirus protein VP16 becomes conformationally constrained upon interaction with basal transcription factors.* J. Biol. Chem., 1996. **271**(9): p. 4827-37.

23. Uesugi, M., et al., *Induced α-helix in the VP16 activation domain upon binding to a human TAF.* Science, 1997. **277**: p. 1310-1313.

24. Kuldell, N.H. and S. Buratowski, *Genetic analysis of the large subunit of yeast transcription factor IIE reveals two regions with distinct functions.* Mol. Cell. Biol., 1997. **17**(9): p. 5288-98.

25. Oliner, J.D., et al., *Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53.* Nature, 1993. **362**(6423): p. 857-60.

26. Van Orden, K., et al., *Binding of p53 to the KIX domain of CREB binding protein.* J. Biol. Chem., 1999. **274**: p. 26321-26328.

27. Choy, B. and M. Green, *Eukaryotic activators function during multiple steps of preinitiation complex assembly.* Nature, 1993. **366**: p. 531-536.

28. Heery, D., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**: p. 733-736.

29. Hu, X. and M. Lazar, *The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors.* Nature, 1999. **402**: p. 93-96.

30. Ko, L.J. and C. Prives, *p53: puzzle and paradigm.* Genes Dev., 1996. **10**(9): p. 1054-72.

31. Kussie, P., et al., *Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain.* Science, 1996. **274**: p. 948-953.

32. McInerney, E.M., et al., *Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation.* Genes Dev., 1998. **12**(21): p. 3357-68.

33. Webb, P., et al., *ERbeta Binds N-CoR in the Presence of Estrogens via an LXXLL-like Motif in the N-CoR C-terminus.* Nucl. Recept., 2003. **1**(1): p. 4.

34. Lee, C., et al., *Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor.* Genes Dev., 2002. **16**: p. 3199-3212.

35. Nguyen, B., et al., *NMR structure of a complex containing the TFIIF subunit RAP74 and the RNA polymerase II carboxyl-terminal domain phosphatase FCP1.* Proc. Natl. Acad. Sci., 2003. **100**: p. 5688-5693.

36. Blau, J., et al., *Three functional classes of transcriptional activation domains.* Mol. Cell. Biol., 1996. **16**: p. 2044-2055.

CHAPTER 3

THE ACIDIC ACTIVATION DOMAIN OF p53 INTERACTS WITH THE IBiD AND KIX DOMAINS OF CBP

Introduction

Cyclic-AMP response element binding protein (CBP) and its yeast homologue p300 are nuclear coactivators that interact with sequence-specific DNA-binding transcription factors. Although there are several functions attributed to CBP/p300, including DNA repair, cell growth, differentiation, and apoptosis, the most important is their regulation of transcriptional events [1]. CBP mediates transcription through interaction with multiple factors, cellular and viral, in response to cellular signals. Mutations in CBP increase the probability of developing disorders such as mental retardation, congenital deformation, and various types of cancers [2].

Human CBP, a large protein consisting of 2441 amino acids, is composed of several functional domains (Figure 3.1). CBP contains three zinc-binding domains, CH1, CH2, and CH3, that have a high number of cysteine and histidine residues. The CH1 and CH3 regions, also termed TAZ1 and TAZ2 respectively, have been shown to interact with both cellular and viral proteins [3]. In the amino-terminus, the KIX domain (amino acids 455-679) is responsible for maintaining the interaction between phosphorylated CREB and its promoter as well as binding other viral and cellular factors such as Tax, BRCA1 and c-Myb [4-7]. The center of the protein contains a bromodomain, that is essential for histone acetyltransferase (HAT) activity [8]. Finally, the IRF-3 binding domain (IBiD) in the carboxy-terminus of CBP has been shown to

directly interact with multiple factors, and is highly conserved throughout several species [9]. Each of the domains present in CBP has the ability to bind factors that regulate transcription. Because CBP is able to interact with over 50 factors, it has the potential to participate in several cellular functions as a coactivator.

The tumor suppressor protein p53 is a cellular transcription factor shown to interact with CBP/p300 through its CH1, KIX, CH3, and IBiD domains [10-13]. In the cytoplasm of unstressed cells, p53 is negatively regulated by the transcription factor MDM2. MDM2 targets p53 for degradation via the ubiquitin-protease pathway [14, 15]. Current research has shown that CBP/p300 may also contribute to regulating the degradation of p53. An indirect role was first observed when the stability of p53 was increased due to the interaction between p300 and adenovirus protein E1A [16]. However, studies have suggested that a complex between p300, MDM2, and p53 is necessary for MDM2-dependent degradation of p53 [10]. Reinforcing this idea, a MDM2 deletion mutant that was able to bind p53 but not p300 was ineffective in inducing p53 degradation.

Post-translational modifications play a critical role in regulating the functions of p53. Although several kinases modify sites throughout the p53 protein, acetylation occurs solely in the carboxy-terminus. Previous studies have shown that CBP acetylates p53 on residues Lys^{373}, Lys^{381}, and Lys^{382} both *in vivo* and *in vitro* [17, 18]. The carboxyl-terminal domain of p53 is important in regulating its sequence-specific DNA-binding activity. Gel shift mobility assays revealed a dramatic 20- to 30-fold increase in the DNA-binding activity of bacterially produced full-length human p53 when acetylated by CBP/p300 [17]. Therefore, CBP contributes to p53 function by acetylating the carboxyl-terminal domain and preventing it from interacting with the DNA-binding domain [19].

CBP/p300 plays an extremely important role in p53-mediated transactivation. To suppress the formation of tumors, p53 activates the transcription of several genes resulting in cell cycle arrest or apoptosis. CBP/p300 has been recognized as a coactivator of p53 and is required for optimal p53 transactivation *in vivo* [17]. It has also been shown that the site of interaction on CBP for this activity is in its carboxyl-terminal domain between residues 1514 and 1737 [20]. Subsequent studies revealed a complex of p53/p300 on DNA as well as verifying that p300 stimulates the transcriptional activity of p53 on p53-regulated promoters [20]. In addition, it has been shown that the transactivation of p53-regulated genes is dependent upon the p53/p300 interaction. When CBP/p300 is inhibited by E1A the transactivation of p53 is also repressed [21].

Previous studies have shown that the AAD of p53 interacts with both the IBiD and KIX domains of CBP [11, 13]. In the present studies, we have further defined the binding site for both the IBiD domain and the KIX domain within the AAD of p53. We have verified that both domains of CBP/p300 do bind within the first 72 amino acids of p53, but that they require different residues. The data lends important insights into the current ideas regarding the relationship between p53 and CBP/p300 and how their interactions contributes to cellular functions.

Materials and Methods

Cloning of recombinant proteins

The p53 deletion mutants used (GST-p53$_{(1\text{-}72,\ 1\text{-}60,1\text{-}40,\ 20\text{-}73)}$) were generously donated by Dr. Rong Li (University of Virginia). The GST-CBP$_{(2055\text{-}2150)}$ was given by the laboratory of Dr. Jennifer

Nyborg (Colorado State University). GST-$CBP_{(588-679)}$ was generously given by Dr. Timothy Osborne (University of California, Irvine). The $p53_{(25-65)}$ peptide used in the 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) experiment was provided by Dr. Ettore Appella (National Institute of Health).

Expression and purification of Glutathione-S- Transferase (GST)-fusion proteins

$CBP_{(2055-2150)}$,$CBP_{(588-679)}$. The GST-$CBP_{(2055-2150)}$ used in the *in vitro* binding experiments was expressed from a pGEX-2T (Amersham) vector as a fusion protein in the *E. coli* host strain TOPP2 (Stratagene). The GST-$CBP_{(588-679)}$ was expressed from the pGEX-3X (Amersham) vector in the DH5α (Invitrogen) cell line. The cells were grown at 37°C and protein expression was induced for 3 hours at 30°C with 0.7 mM isopropyl-D-thiogalactoside (IPTG) (Inalco). The cells were harvested by centrifugation and resuspended in EBC buffer (50 mM Tris-Cl pH 8.0, 120 mM NaCl, 0.5% NP-40, 2 mM DTT). The cells were then lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatant from the centrifugation was incubated for 1 hour with 3 mL of Glutathione Sepharose (GSH) resin (Amersham) at 4°C. Following incubation, the resin was collected by centrifugation and washed twice with NETN buffer (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.05% SDS, 1 mM DTT) and once in phosphate-buffered saline PBS pH 7.4 (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). $CBP_{(2055-2150)}$ was eluted off the resin by rotating for ten minutes in Elution buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM DTT, 15 mM reduced Glutathione). $CBP_{(2055-2150)}$ was then dialyzed into Freezing buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 20% glycerol, 2 mM DTT) and stored at -80°C. The $CBP_{(588-679)}$ resin was diluted 1:1 with NETN buffer and kept at -80°C for future use in *in vitro* binding assays.

Protein expression and purification

$p53_{(1-72)}$,$p53_{(1-60)}$, $p53_{(1-40)}$, $p53_{(20-73)}$. p53 fragments$_{(1-72,1-60,1-40,\ 20-73)}$ were expressed from the pGEX-2TK (Amersham) vector as a GST-fusion protein in *E. coli* host strain TOPP2. The cells were grown overnight at 37°C, and protein expression was induced for 4 hours with 0.7 mM IPTG at 30°C. The cells were harvested by centrifugation, resuspended in GST lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 M NaCl, 1 mM DTT), lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatants were incubated for 1 hour with 10mL of GSH resin at 4°C. Following incubation, the resins were washed with lysis buffer three times followed by equilibration with PBS pH 7.4 containing 1 mM DTT. The resins containing the bound fusion proteins were then incubated overnight at room temperature with 100 units of thrombin (Calbiochem) to cut the $p53_{(1-72)}$, $p53_{(1-60)}$, $p53_{(1-40)}$, and $p53_{(20-73)}$ fragments from the GST. Following thrombin cleavage, the supernatants were incubated for 1 hour with Benzamidine Sepharose (Pharmacia), filtered to remove the Benzamidine Sepharose, dialyzed into buffer A (20 mM phosphate buffer pH 6.5, 1 mM DTT, 1mM EDTA), and then applied to a Q-Sepharose High Performance (Pharmacia) column (75 mL) equilibrated with buffer A. The $p53_{(1-72)}$, $p53_{(1-60)}$, $p53_{(1-40)}$, and $p53_{(20-73)}$ fragments were eluted from the column using a gradient (from 0 to 100% over 700 mL) of buffer B (20 mM phosphate buffer, 1 mM DTT, 1 mM EDTA, and 1M NaCl). Fractions containing the purified $p53_{(1-72)}$, $p53_{(1-60)}$, $p53_{(1-40)}$, and $p53_{(20-73)}$ fragments were then pooled, dialyzed overnight into buffer A, and concentrated for *in vitro* binding analysis.

(4) $CBP_{(2055-2150)}$ for NMR Spectroscopy. $CBP_{(2055-2150)}$ was expressed from the pGEX-2T vector as a GST-fusion protein in *E. coli* host strain TOPP2. The cells were grown overnight at 37°C, and protein expression was induced for 4 hours with 0.7 mM IPTG at 30°C. The cells

were harvested by centrifugation, resuspended in GST lysis buffer, lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatants were incubated for 1 hour with 10 mL of GSH resin at 4°C. Following incubation, the resins were washed with lysis buffer three times followed by equilibration with PBS pH 7.4 containing 1 mM DTT. The resins containing the bound fusion proteins were then incubated for 2 hours at room temperature with 100 units of thrombin to cut the $CBP_{(2055-2150)}$ fragment from the GST. Following thrombin cleavage, the supernatants were incubated for 1 hour with Benzamidine Sepharose, filtered to remove Benzamidine Sepharose, dialyzed into buffer A, and then applied to a S-Sepharose High Performance column (Pharmacia) (75 mL) equilibrated with buffer A. The $CBP_{(2055-2150)}$ fragment was eluted from the column using a gradient (from 0 to 100% over 700 mL) of buffer B. Fractions containing the purified $CBP_{(2055-2150)}$ fragment were then pooled, dialyzed overnight into buffer A, and concentrated for NMR spectroscopy. Uniform (>98%) ^{15}N labeling was performed by growing the cells in a modified minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The purification was carried out as described for the unlabeled $CBP_{(2055-2150)}$ fragment.

***In Vitro* Binding Assays.**

All *in vitro* binding experiments were performed using 12.5 µl of GSH resin in 0.5X Superdex buffer (25 mM Hepes, pH 7.9, 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 150 mM KCl , 20% glycerol, 0.1% Nonidet P-40, 1 mM EDTA). For each assay, 1 µM of Glutathione-S-Transferase (GST), or GST-$CBP_{(2055-2150)}$ fusion protein was incubated with the GSH resin for 1 hour at 4°C with constant rotation. The 1 µM GST-$CBP_{(588-679)}$ was not eluted therefore, we did not need to rebind it to the resin. The resin was then washed two times with 0.5 mL of 0.5X Superdex buffer.

Purified p53 proteins were then added at various concentrations (0.1 μM-1 μM) to the washed resin in 0.5 mL of 0.5X Superdex buffer and incubated for 1 hour at 4°C with constant rotation. The resin was then washed twice as before with 0.5 mL of 0.5X Superdex buffer. Following the wash step, 25 μl of Elution Buffer (100 mM Tris-HCl pH 6.8, 140 mM SDS, 200 mM DTT, 2.3% Bromophenol Blue, 20% glycerol) was added to elute the bound protein. The samples were heated for 10 minutes at 60°C in elution buffer and spun down in a centrifuge to pellet the resin. The supernatant (20 μl) was loaded onto a 15% acrylamide gel and separated by electrophoresis using a Tris-Glycine buffer (10% Tris-Glycine[1.92 M Glycine, 0.25 M Tris], 10% SDS). The proteins were then transferred to an Immobilon-P membrane (Millipore) at 4°C in 1X Tris-Glycine for 1 hour at 44V. The membrane was incubated overnight in 50 mL of 1X TBS/Blotto (100 mM Tris-Cl pH 8.0, 1.5 M NaCl, 5% nonfat dry milk) with constant shaking. The following day the membrane was incubated in 10mL of TBST/Blotto (100 mM Tris-Cl pH 8.0, 1.5 M NaCl, 0.05% Tween 20, 5% nonfat dry milk) and a 1:10000 dilution of an anti-p53 primary antibody (p53 DO1[epitope 11-35] or Pab 1801[epitope 32-79]; Santa Cruz) for 1 hour. Excess primary antibody was removed by three successive 5-minute washes with TBST. The washed membrane was then incubated in 10 mL of TBST/Blotto and a 1:5000 dilution of secondary antibody (goat-antimouse IgG-HRP sc-2055; Santa Cruz) for 30 minutes. The membrane was washed four times with TBST for five minutes to remove the excess secondary antibody. The protein was detected by autoradiography through chemiluminescence using ECL Plus (Amersham). The membrane was Coomassie stained to ensure equal molar concentrations of protein inputs for the GST control and the GST-fusion proteins.

Results

The IBiD domain of CBP interacts with the AAD of p53

One mechanism by which CBP functions as a coactivator is by stimulating p53 transactivation [17]. This is accomplished through one of the multiple p53 interaction sites on CBP. Using GST pull-down and electrophoretic mobility shift assays, it has been shown that a fragment containing the AAD of p53 ($p53_{(1-107)}$) binds the IBiD domain of CBP [13]. Therefore, we wanted to examine whether the AAD of p53 was responsible for this interaction. First, purified recombinant $p53_{(1-72)}$ was incubated with GST-$CBP_{(2055-2150)}$ in a GST pull-down assay to determine if it was sufficient for binding (Figure 3.2). Results revealed that the AAD of p53 interacts specifically with the IBiD domain of CBP.

The minimal binding domain of the IBiD domain of CBP lies between residues 25 and 65 of p53

Once it was determined that the AAD of p53 binds the IBiD domain of CBP, we wanted to define the minimal binding domain of p53 required for the interaction. We first purified deletion mutants of the p53 $AAD_{(1-40,1-60,20-73)}$ to assay their ability interact with $CBP_{(2055-2150)}$. The first mutant analyzed, $p53_{(1-40)}$, has been shown to be sufficient for interaction with several proteins most notably MDM2 [11, 22-24]. Therefore, GST-$CBP_{(2055-2150)}$ was incubated with various concentrations of $p53_{(1-40)}$ in a GST pull-down assay (Figure 3.2*)*. This experiment demonstrated that $p53_{(1-40)}$ was not capable of binding $CBP_{(2055-2150)}$ when compared to the $p53_{(1-72)}$ fragment under the same conditions. This suggests that either $p53_{(1-40)}$ is not required for binding or that the entire AAD of p53 is needed for interaction with the IBiD domain. We were next interested in determining whether $p53_{(20-73)}$, would be sufficient for binding as seen with

Tfb1 and p62. Like the $p53_{(1-40)}$ fragment, $p53_{(20-73)}$ was incubated with $CBP_{(2055-2150)}$ to determine if it was sufficient for binding (Figure 3.3). Based on this result, $p53_{(20-73)}$ binds $CBP_{(2055-2150)}$ with the same affinity as the $p53_{(1-72)}$ fragment.

After determining the site of interaction between the IBiD domain and p53 is most likely in AAD2, we wanted to examine what amino acids represented the amino- and carboxyl-terminal borders. The first construct chosen was $p53_{(1-60)}$ because it lacks twelve residues present in the carboxy-terminus of the $p53_{(20-73)}$ fragment. Various concentrations of $p53_{(1-60)}$ were incubated with GST-$CBP_{(2055-2150)}$ in a GST pull-down assay. We observed that while there is binding to the $p53_{(1-60)}$ fragment, it binds significantly weaker than $p53_{(1-72)}$ (Figure 3.4). Based on these results, it appears there are residues between amino acids 60 and 72 of p53 that are important for interaction with the IBiD domain of CBP/p300.

In order to test this hypothesis and further determine the minimal region of p53 required for interaction, a peptide was chemically synthesized ($p53_{(25-65)}$) to analyze complex formation using NMR. This peptide was chosen because it lacks five amino-terminal residues present in the amino-terminus of the $p53_{(20-73)}$ fragment but replaces five amino acids not present in the carboxy-terminus of the $p53_{(1-60)}$ construct. A 2D ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) experiment was performed by titrating 1.2 mM unlabeled $p53_{(25-65)}$ into a sample containing 1.2 mM ^{15}N-labeled $CBP_{(2055-2150)}$. An overlay of the ^{1}H-^{15}N HSQC spectra of free ^{15}N-labeled $CBP_{(2055-2150)}$ (black) and of the ^{15}N-labeled $CBP_{(2055-2150)}$/$p53_{(25-65)}$ (red) showed considerable changes (Δ (^{1}H) $\geq$ 0.05 ppm and/or Δ (^{15}N) $\geq$ 0.5 ppm) in both ^{1}H and ^{15}N chemical shifts indicating complex formation. Collectively, these data show that the minimal region of p53 capable of binding the IBiD domain of CBP lies between residues 25 and 65.

The AAD of p53 interacts with the KIX and IBiD domains of CBP through a similar binding site

Another region of CBP also shown to bind the AAD of p53 is the KIX domain ($CBP_{588-679}$) [11]. Based on the previous study we were interested in whether the IBiD and KIX domains share a common binding site. We first wanted to duplicate previous results using our purified $p53_{(1-72)}$ fragment. As with the IBiD domain, the KIX domain ($CBP_{588-679}$) was expressed as a GST-fusion protein and incubated with $p53_{(1-72)}$ in a GST pull-down assay. As expected, $CBP_{(588-679)}$ interacts specifically with the AAD of $p53_{(1-72)}$ (Figure 3.6).

Previous studies have shown, that an antibody directed against residues in AAD1 of p53 inhibited interaction with the KIX domain of CBP/p300 in a competition assay [11]. In the same study, a double point mutation in p53 (L22Q/W23S) negatively affected binding to the KIX domain of CBP/p300 in a GST pull-down assay. Since the L22Q/W23S mutant disrupted interaction with the KIX domain, it was assumed that the binding site for the KIX domain of CBP/p300 on p53 was the same as the binding site for MDM2 (residues 1-40). Interestingly, when $CBP_{(588-679)}$ was incubated with $p53_{(1-40)}$ in a GST pull-down assay, an interaction was not observed suggesting that perhaps either AAD2 or the entire AAD is required for binding (Figure 3.6). To further investigate this hypothesis, $CBP_{(588-679)}$ was next incubated with $p53_{(1-60)}$ to determine if the addition of twenty amino acids to the carboxy-terminus restored the interaction. Results showed $CBP_{(588-679)}$ interacts with $p53_{(1-60)}$ with a higher affinity than the $p53_{(1-40)}$ fragment re-establishing binding to an affinity similar to that obtained with the $p53_{(1-72)}$ fragment (Figure 3.7). Based on these results, it appears that the addition of residues 41 to 60 present within AAD2 contribute significantly to the interaction between p53 and the KIX domain of CBP. Finally, $CBP_{(588-679)}$ was assayed with the $p53_{(20-73)}$ fragment to determine if the KIX required the first twenty amino acids, since these amino acids were not required for interaction

with the IBiD domain of CBP. Interestingly, the results demonstrated that $p53_{(20-73)}$ is not capable of binding the KIX domain. Based on these results, we propose that although the two domains bind to p53 within the first seventy-two amino acids, their binding sites are not identical.

Discussion

The transcription of genes is regulated in part by the communication between activators, coactivators, and their DNA targets. In the cell, CBP contributes to the regulation of p53 through post-translational modifications, while mediating its degradation and transcriptional activity [10, 17, 18]. The regulation of p53 by CBP is most likely accomplished through multiple p53 interaction sites present on CBP**.** In this study, we used *in vitro* binding assays and NMR spectroscopy to identify the region in the amino-terminus of p53 required for interaction with the IBiD and KIX domains of CBP. The AAD of p53 has been shown to interact with both the KIX and IBiD domains of CBP [11, 17]. Our goal was to continue mapping the binding site on the p53 protein using, as a starting point the full AAD of p53 ($p53_{(1-72)}$).

When interaction between the IBiD domain of CBP and p53 was first reported, the p53-binding region on CBP was precisely mapped between amino acids 2058 and 2130. This was further established when a double point mutation introduced in the IBiD domain (L2068A / L2071A) disrupted key structural elements resulting in a decrease in binding to p53 [13]. CBP/p300 interacts with several other proteins through this region including IRF-3, SRC-1, and E1A [9, 25]. Deletion mutants allowed us to observe whether residues in the entire AAD ($p53_{(1-72)}$), AAD1 ($p53_{(1-40)}$), or AAD2 ($p53_{(40-83)}$) are required for this interaction. As previously

stated, amino acids 13-23 of AAD1 are conserved across several species. In the x-ray structure of the p53 / MDM2 complex, residues Phe^{19}, Trp^{23}, and Leu^{26} of AAD1 are critical for binding. Therefore, if the AAD1 subdomain is responsible for interaction with the IBiD and KIX domains, the $p53_{(1-40)}$ fragment should be sufficient as was the case for MDM2. Our results revealed that while the AAD of p53 is responsible for interaction with the IBiD and KIX domains, AAD1 ($p53_{(1-40)}$) is not sufficient for binding to either the KIX or IBiD domain (Figure 3.2 and 3.6). For the IBiD domain, this data coincides with previous research revealing that a double point mutation (L22Q / W23S) in p53 did not result in a decrease in binding as seen with MDM2 [13]. On the other hand, our findings seem to be inconsistent with results showing that the same double mutation significantly decreased the binding of p53 to the KIX domain [11]. Taken together, these two results suggest that AAD1 is not sufficient for interaction with the KIX domain, but it contains important elements required for binding.

Once it was determined that amino acids 1 through 40 were not sufficient for binding to either the IBiD or KIX domains, a construct was purified that extended to residue 60 of p53 ($p53_{1-60)}$). *In vitro* binding assays were then used to analyze whether the extra twenty residues in this fragment would restore binding affinity to that observed with the $p53_{(1-72)}$ fragment. The binding of $p53_{(1-60)}$ to the IBiD domain of CBP was observed, however, when compared to $p53_{(1-72)}$ under the same conditions, binding was significantly weaker. This suggests that the binding region for the IBiD domain extends far into the carboxyl-terminal end of AAD2 (Figure 3.3). Results with the KIX domain demonstrated that $p53_{(1-60)}$ re-establishes binding to an affinity similar to that observed with $p53_{(1-72)}$. Therefore, the KIX domain appears to interact with residues in AAD2 of p53 between amino acids 40 and 60.

Finally, a construct was purified that removed the first twenty amino acids of the AAD of p53 ($p53_{(20-73)}$). Incubation of $p53_{(20-73)}$ with the IBiD domain resulted in an increase in binding when compared to the $p53_{(1-40)}$ and $p53_{(1-60)}$ fragments (Figure 3.4). Based upon results showing the $p53_{(20-73)}$ fragment re-established binding to nearly the same affinity as $p53_{(1-72),}$ it appears that residues required for interaction with the IBiD domain lie between amino acids 60 to 73, but the first twenty amino acids are not required. In order to verify this hypothesis using a different biochemical method, NMR studies were performed with a peptide containing amino acids 25 to 65 of p53 in complex with the IBiD domain. This same peptide was shown in the previous chapter to be sufficient for binding to the amino-terminal domain of Tfb1. In agreement with the *in vitro* binding studies, the ^{1}H-^{15}N HSQC experiment showed complex formation suggesting that the binding domain required for interaction with $CBP_{(2055-2150)}$ lies between amino acids 25 and 65 of p53. This is identical to the domain required for binding to Tfb1 and very similar but not identical to the region required for interaction with the KIX domain.

Further research, such as structural studies or point mutations, will be needed in order to determine the exact binding interface for p53 with both the IBiD and KIX domains of CBP. However, it is clear that both of these fragments bind in a manner different than what has been seen in the p53 / MDM2 complex. Based on our results, it can be said that critical residues required for the p53 / IBiD domain interaction are located between amino acids 60 and 65, and the first twenty amino acids are not required. In addition, residues critical for the p53 / KIX site are found entirely within the first 60 amino acids and the first twenty amino acids are important (Figure 3.8) [11]. Thus, the KIX domain and the IBiD domain of CBP bind to slightly different sites within the AAD of p53.

Biological implications of the interaction of p53 with the IBiD and KIX domains of CBP on transcription have been previously researched. It has been shown through *in vitro* studies that the interaction of p53 with the IBiD domain of CBP stimulates the rate of transcription [13]. It is believed that the protein Tax may play a role in the regulation of this activation. Although the exact mechanism is unknown, competition between Tax and p53 for CBP may explain how the expression of Tax inhibits the transcriptional activation of p53 [26, 27]. The mechanism by which this occurs is not known, but it could be due to competing for the same sites on CBP. Previous studies have shown that Tax decreases the amount of p53 bound to the IBiD domain [13]. In addition, Tax and p53 also compete for the KIX domain, which in turn inhibits p53-mediated transcription [11]. Therefore, Tax affects the ability of p53 to activate transcription by preventing its contact with CBP. Further determination of how these interactions are mediated will lend important insight on the intricate interplay between p53, coactivator CBP, and their effect on cellular functions.

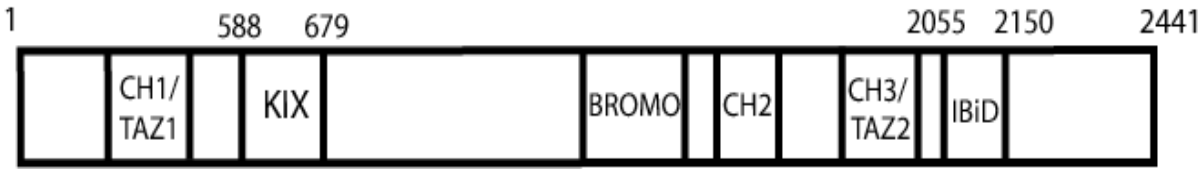


Figure 3.1. Schematic representation of the mammalian coactivator CBP depicting its functional domains

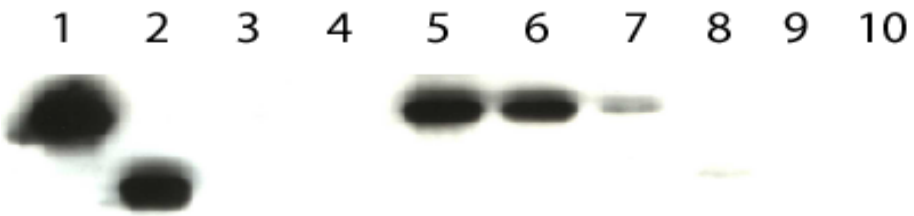


Figure 3.2. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(1-40)}$ to the IBID domain of CBP. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-40)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(2055-2150)}$. In the GST lanes, 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(1-40)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(1-40)}$ (lane 2).

Figure 3.3.. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(20-73)}$ to the IBID domain of CBP. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(20-73)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(2055-2150)}$. In the GST lanes, 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(20-73)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(20-73)}$ (lane 2).

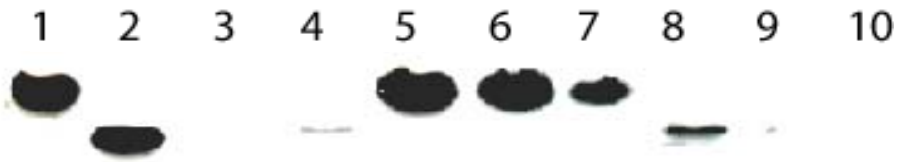


Figure 3.4. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(1-60)}$ to the IBID domain of CBP. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-60)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(2055-2150)}$. In the GST, lanes 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(1-60)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(1-60)}$ (lane 2).

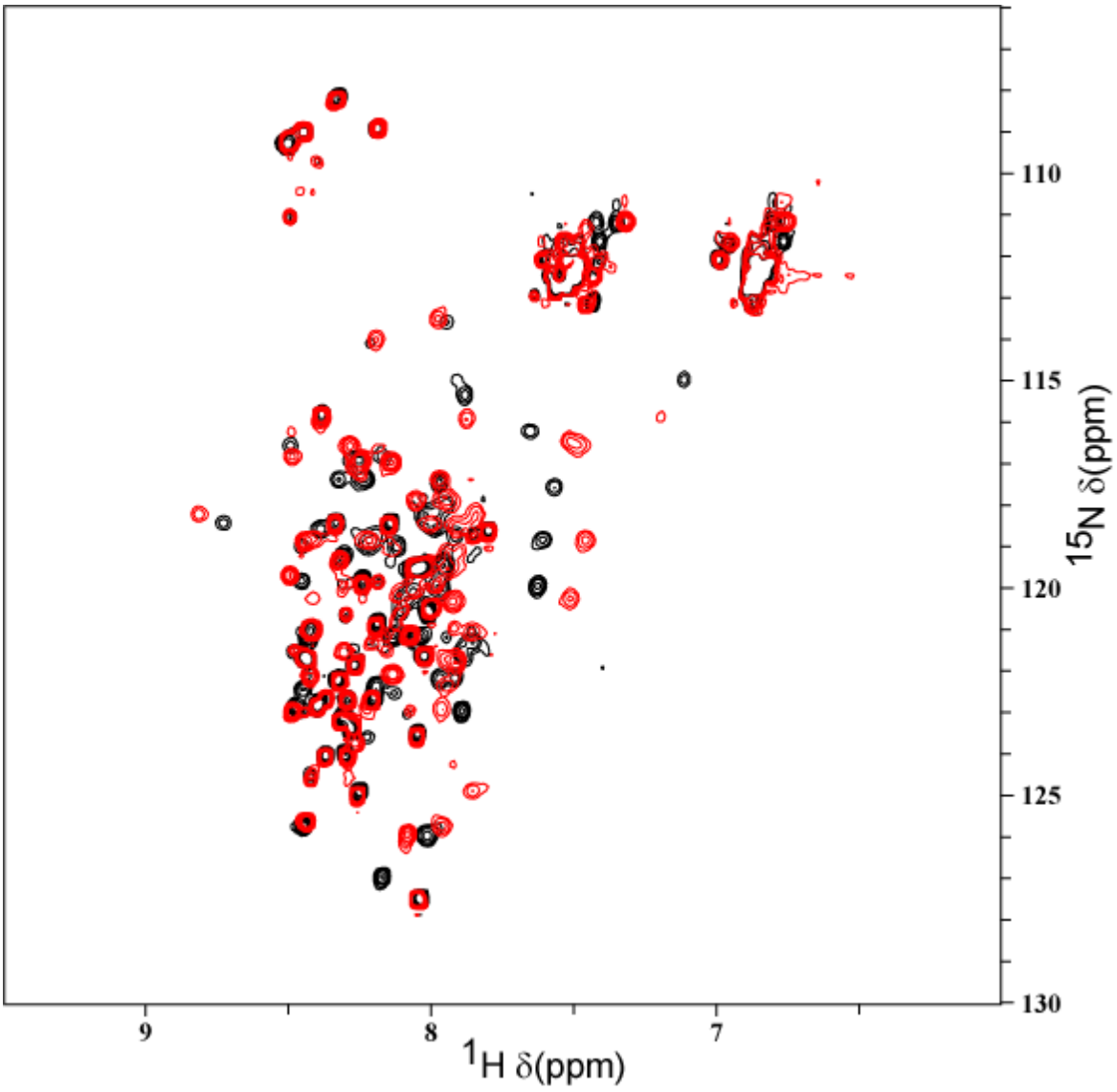


Figure 3.5. Overlay of the 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled $CBP_{(2055-2150)}$ in the absence (black) and presence (red) of unlabeled $p53_{(25-65)}$.

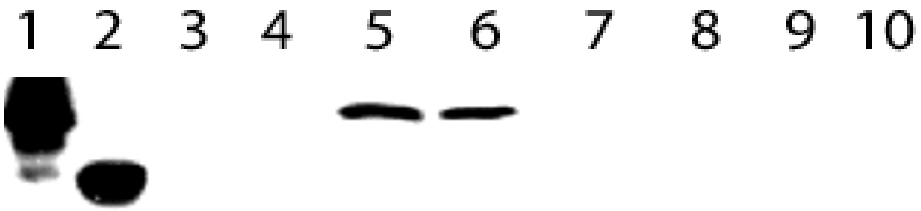


Figure 3.6. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(1-40)}$ to the KIX domain of CBP. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-40)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(588-679)}$. In the GST lanes, 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(1-40)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(1-40)}$ (lane 2).

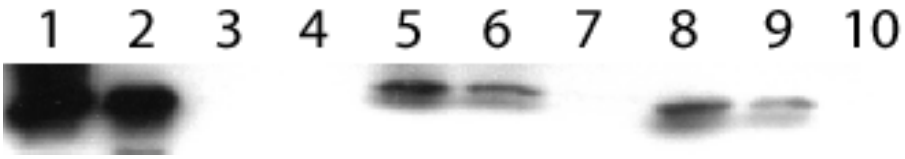


Figure 3.7. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(1-60)}$ to the KIX domain of CBP. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(1-60)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(588-679)}$. In the GST lanes, 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(1-60)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(1-60)}$ (lane 2).

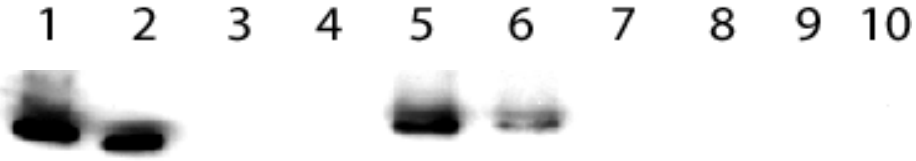


Figure 3.8. Comparison of the *in vitro* binding of $p53_{(1-72)}$ and $p53_{(20-73)}$ to the KIX domain of CBP.. Varying concentrations (1.0, 0.5, 0.1 μM) of $p53_{(1-72)}$ (lanes 5-7) and $p53_{(20-73)}$ (lanes 8-10) were incubated with 1 μM GST-$CBP_{(588-679)}$. In the GST lanes, 1 μM of purified $p53_{(1-72)}$ (lane 3) and $p53_{(20-73)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% $p53_{(1-72)}$ (lane 1) and $p53_{(20-73)}$ (lane 2).

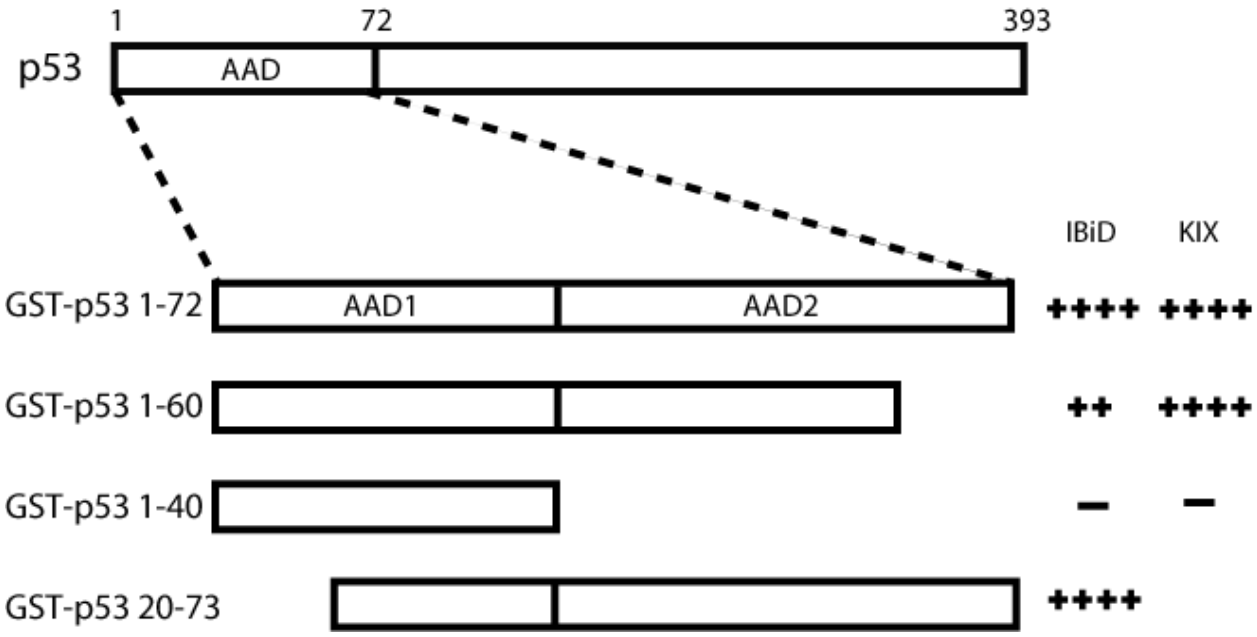


Figure 3.9. Deletion mutants of the p53 AAD and a summary of their binding affinity to the IBiD and KIX domains of CBP.

References

1. Goodman, R.H. and S. Smolik, *CBP/p300 in cell growth, transformation, and development.* Genes Dev., 2000. **14**(13): p. 1553-77.

2. Giles, R.H., D.J. Peters, and M.H. Breuning, *Conjunction dysfunction: CBP/p300 in human disease.* Trends Genet., 1998. **14**(5): p. 178-83.

3. De Guzman, R.N., et al., *Solution structure of the TAZ2 (CH3) domain of the transcriptional adaptor protein CBP.* J. Mol. Biol., 2000. **303**(2): p. 243-53.

4. Parker, D., et al., *Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism.* Mol. Cell. Biol., 1996. **16**(2): p. 694-703.

5. Giebler, H.A., et al., *Anchoring of CREB binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation.* Mol. Cell. Biol., 1997. **17**(9): p. 5156-64.

6. Campbell, K.M. and K.J. Lumb, *Structurally distinct modes of recognition of the KIX domain of CBP by Jun and CREB.* Biochemistry, 2002. **41**(47): p. 13956-64.

7. Dai, P., et al., *CBP as a transcriptional coactivator of c-Myb.* Genes Dev., 1996. **10**(5): p. 528-40.

8. Deng, Z., et al., *The CBP bromodomain and nucleosome targeting are required for Zta-directed nucleosome acetylation and transcription activation.* Mol. Cell. Biol., 2003. **23**(8): p. 2633-44.

9. Lin, C.H., et al., *A small domain of CBP/p300 binds diverse proteins: solution structure and functional studies.* Mol. Cell, 2001. **8**(3): p. 581-90.

10. Grossman, S.R., et al., *p300/MDM2 complexes participate in MDM2-mediated p53 degradation.* Mol. Cell, 1998. **2**(4): p. 405-15.

11. Van Orden, K., et al., *Binding of p53 to the KIX domain of CREB binding protein.* J. Biol. Chem., 1999. **274**: p. 26321-26328.

12. O'Connor, M.J., et al., *Characterization of an E1A-CBP interaction defines a novel transcriptional adapter motif (TRAM) in CBP/p300.* J. Virol., 1999. **73**(5): p. 3574-81.

13. Livengood, J.A., et al., *p53 Transcriptional activity is mediated through the SRC1-interacting domain of CBP/p300.* J. Biol. Chem., 2002. **277**(11): p. 9054-61.

14. Levine, A.J., *p53, the cellular gatekeeper for growth and division.* Cell, 1997. **88**(3): p. 323-31.

15. Momand, J., et al., *The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation.* Cell, 1992. **69**(7): p. 1237-45.

16. Querido, E., J.G. Teodoro, and P.E. Branton, *Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis.* J. Virol., 1997. **71**(5): p. 3526-33.

17. Gu, W. and R. Roeder, *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell, 1997. **4**: p. 595-606.

18. Sakaguchi, K., et al., *DNA damage activates p53 through a phosphorylation-acetylation cascade.* Genes Dev., 1998. **18**: p. 2831-2841.

19. Ko, L.J. and C. Prives, *p53: puzzle and paradigm.* Genes Dev., 1996. **10**(9): p. 1054-72.

20. Avantaggiati, M.L., et al., *Recruitment of p300/CBP in p53-dependent signal pathways.* Cell, 1997. **89**(7): p. 1175-84.

21. Lill, N.L., et al., *Binding and modulation of p53 by p300/CBP coactivators.* Nature, 1997. **387**(6635): p. 823-7.

22. Thut, C., et al., *p53 transcriptional activation mediated by coactivators $TAF_{II}40$ and $TAF_{II}60$.* Science, 1995. **267**: p. 100-104.

23. Uesugi, M., et al., *Induced α-helix in the VP16 activation domain upon binding to a human TAF.* Science, 1997. **277**: p. 1310-1313.

24. Oliner, J.D., et al., *Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53.* Nature, 1993. **362**(6423): p. 857-60.

25. Sheppard, H.M., et al., *Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1.* Mol. Cell. Biol., 2001. **21**(1): p. 39-50.

26. Akagi, T., et al., *Aberrant expression and function of p53 in T-cells immortalized by HTLV-1 Tax1.* FEBS Lett., 1997. **406**(3): p. 263-266.

27. Mulloy, J., et al., *Human T-cell lymphotrophic/leukemia virus type I Tax abrogates p53-induced cell cycle arrest and apoptosis through its CREB/ATF functional domain.* J. Virol., 1998. **72**(11): p. 8852-8860.

CHAPTER 4

THE AAD OF p53 INTERACTS WITH THE ZINC-BINDING DOMAINS OF GATA-1 AND GATA-2

Introduction

Members of the GATA transcription factor family are zinc-binding domain (ZBD) containing proteins found in a wide range of organisms from unicellular to vertebrates. GATA factors recognize the consensus DNA sequence (T/A)GATA(A/G) [1]. In vertebrates, six GATA factors have been characterized according to structural homology and tissue distribution. GATA-1, -2, and –3, 'hematopoietic' GATA factors, are expressed in tissues which are responsible for the formation of blood and blood cells [2-4]. Numerous studies have shown that GATA-1 and GATA-2 play an important role in gene regulation during the development and differentiation of red blood cells [8]. The remaining three factors, GATA-4, -5,and –6 are termed 'endodermal' because they are found in cells associated with organ development such as the heart, gut, lung, and liver [5-7].

The ZBDs present in all GATA factors are very highly conserved. In vertebrates, there are two of these domains present with the consensus sequence Cys-X_2-Cys-X_{17}-Cys-X_2-Cys and they are referred to as the amino-terminal ZBD (NZ) and the carboxyl-terminal ZBD (CZ) [9]. Immediately, adjacent to the CZ is a region consisting of several basic amino acids known as the basic arm (BA). Functions associated with the CZ

include DNA-binding and nuclear localization activity [10]. In addition, the solution structure of a complex between the DNA-binding domain (DBD) of GATA-1 and a 16-base pair DNA sequence has been determined [11]. In this structure, it was revealed that both the CZ and the BA were essential for specific interaction with the target GATA containing DNA target. Interestingly, the CZ and the BA are the only regions completely conserved throughout the entire GATA family [9]. The NZ possesses a region capable of transcriptional activation and is required for interaction with cofactors or other transcription factors [10, 12]. This was first revealed when the NZ of GATA fused to a heterologous DBD activated transcription [13]. The NZ not only stimulates the activation of transcription directly but also indirectly through interaction with other cofactors such as CBP/p300 and Friend of GATA-1 (FOG-1) [12].

GATA-1, the first factor cloned and characterized, is found in both hematopoietic cells and the Sertoli cells of the testis [8, 14]. Strictly regulated, it is required for the survival and differentiation of erythroid cells as well as controlling testicular development. GATA-1 accomplishes its function by regulating gene expression typically through interaction with other activators such as FOG-1 although it possesses an activation domain [15]. It has been shown that GATA-1 deficient cells can potentially lead to tumor formation or undergo apotosis [16-18].

In the cell, the activation of genes by GATA-1 is maintained in cooperation with another GATA-family member, GATA-2. In mutant cells lacking GATA-1, the transcription of GATA-2 is upregulated by approximately 50-fold [18]. Although these cells usually do not survive, GATA-2 attempts to overcome the loss of GATA-1. GATA-2 also functions in hematopoietic cells by regulating the maintenance and proliferation of

progenitors [19]. For example, mouse embryos deficient in GATA-2 die in approximately 10 to 11 days due to anemia [3]. In addition, cells lacking GATA-2 introduced into wild-type blastocysts result in daughter cells containing several defects [3]. GATA-2 is also required for the generation of mast cells. Cells generated without GATA-2 and prevented from undergoing apoptosis by the absence of p53, revealed that while the development of some cells such as erythroids still continued, a deficiency in mast cells was observed [19].

Another transcription factor expressed specifically in hematopoietic tissues, PU.1, inhibits erythroid differentiation causing erythroleukemia in mice [20]. PU.1 interacts specifically with GATA-1 *in vivo* and inhibits its ability to activate transcription. PU.1 can interact with the ZBD of GATA-1 using either its amino-terminal acidic activation domain (AAD) or its carboxyl-terminal DNA-binding domain. Although the binding interface of PU.1 and GATA-1 resides in both domains, the transactivation domain of PU.1 is required to inhibit GATA-1 transcriptional activity [21]. The transactivation domain of PU.1 contains two types of activation domains essential for function, acid- and glutamine-rich [22]. The AAD, residues 7 to 74, contains numerous phosphorylation sites and is very similar to the AAD of tumor suppressor protein p53. Therefore, we investigated the possibility that the AAD of p53 interacts with the ZBDs of GATA-1 in a similar manner. Through the use of *in vitro* binding assays, we determined that the AAD of p53 specifically interacts with the ZBDs of both GATA-1 and GATA-2.

Cloning of recombinant proteins

The p53 deletion mutants used (GST-p53$_{(1\text{-}72,\ 1\text{-}40,\ 20\text{-}73)}$) were generously donated by Dr. Rong Li (University of Virginia). The GATA-1 deletion mutants used, (GST-GATA-1$_{(199\text{-}317,\ 200\text{-}251,\ 252\text{-}317,\ 228\text{-}317)}$) , were generously donated by Dr. Cecelia Trainor (National Institute of Health). The GST-GATA-2$_{(280\text{-}412)}$ clone, was given by the laboratory of Dr. David Gordon (University of Colorado Health Science Center).

Expression and purification of Glutathione-S-Transferase (GST)-fusion proteins

GATA-1$_{(199\text{-}317)}$,GATA-1$_{(200\text{-}251),}$ GATA-1$_{(252\text{-}317),}$ GATA-1$_{(228\text{-}317),}$GATA-2$_{(280\text{-}412)}$.

The GST-GATA-1 $_{(199\text{-}317,228\text{-}317)}$ and GST-GATA-2$_{(280\text{-}412)}$ fragments were expressed as fusion proteins in the *E. coli* host strain BL21(DE3) (Novagen). The GST-GATA-1$_{(200\text{-}251,252\text{-}317)}$ were expressed in TOPP2 cells (Stratagene). All GATA-1 fragments were inserted into a pGEX-5X (Amersham) vector and GATA-2 into a pGEX-2TK (Amersham) vector. The cells were grown at 37°C and protein expression was induced for 3 hours at 30°C with 0.7 mM isopropyl-D-thiogalactoside (IPTG) (Inalco). The cells were harvested by centrifugation and resuspended in EBC buffer (50 mM Tris-Cl pH 8.0, 120 mM NaCl, 0.5% NP-40, 2 mM DTT). The cells were then lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatant from the centrifugation was incubated for 1 hour with 3 mL of GSH resin at 4°C. Following incubation, the resin was collected by centrifugation and washed twice with NETN buffer (20mM Tris-Cl pH 8.0, 100 mM NaCl, 1mM EDTA, 0.5% NP-40, 0.05% SDS, 1mM DTT) and once in phosphate-buffered saline (PBS) pH 7.4 (140 mM NaCl, 3

mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The resin was diluted 1:1 with NETN buffer and kept at -80°C for future use in *in vitro* binding assays.

Protein expression and purification

$p53_{(1-72)}$,$p53_{(1-60)}$, $p53_{(1-40)}$, $p53_{(20-73)}$. GST-p53 fragments$_{(1-72,1-60,1-40,\ 20-73)}$ were expressed from the pGEX-2TK vector as a GST-fusion protein in *E. coli* host strain TOPP2. The cells were grown overnight at 37°C, and protein expression was induced for 4 hours with 0.7 mM IPTG at 30°C. The cells were harvested by centrifugation, resuspended in GST lysis buffer (20 mM Tris-HCl pH 7.4, 1mM EDTA, 1M NaCl, 1mM DTT), lysed by passage through a French press, and centrifuged at 100,000 g for 45 minutes. The supernatants were incubated for 1 hour with 10mL of Glutathione-Sepharose (GSH) resin (Amersham) at 4°C. Following incubation, the resins were washed with lysis buffer three times followed by equilibration with phosphate buffered saline PBS pH 7.4 containing 1 mM DTT. The resins containing the bound fusion proteins were then incubated overnight at room temperature with 100 units of thrombin (Calbiochem) to cut the $p53_{(1-72)}$, $p53_{(1-60)}$, $p53_{(1-40)}$, $p53_{(20-73)}$ fragments from the GST. Following thrombin cleavage, the supernatants were incubated for 1 hour with Benzamidine Sepharose (Amersham), filtered to remove the Benzamidine Sepharose, dialyzed into buffer A (20 mM phosphate buffer pH 6.5, 1 mM DTT, 1mM EDTA), and then applied to a Q-Sepharose High Performance (Amersham) column (75 mL) equilibrated with buffer A. The $p53_{(1-72)}$,$p53_{(1-60)}$, $p53_{(1-40)}$, $p53_{(20-73)}$ fragments were eluted from the column using a gradient (from 0 to 100% over 700 mL) of buffer B (20 mM phosphate buffer, 1 mM DTT, 1 mM EDTA, and 1M NaCl). Fractions containing

the purified $p53_{(1-72)}$,$p53_{(1-60)}$, $p53_{(1-40)}$, $p53_{(20-73)}$ fragments were then pooled, dialyzed overnight into buffer A, and concentrated for *in vitro* binding analysis.

In Vitro Binding Assays.

All *in vitro* binding experiments were performed using 12.5 µl of GSH resin in 0.5X Superdex buffer (25mM Hepes, pH 7.9, 12.5mM $MgCl_2$, 10µM $ZnSO_4$, 150mM KCl , 20% glycerol, 0.1% Nonidet P-40, 1mM EDTA). For each assay, 1 µM of Glutathione-S-Transferase (GST), GST-$GATA1_{(199-317,\ 200-251,\ 252-317,\ 228-317)}$ or $GATA-2_{(280-412)}$ fusion protein was incubated with the GSH resin for 1 hour at 4°C with constant rotation. The resin was then washed two times with 0.5 mL of 0.5X Superdex buffer. Purified p53 was then added at various concentrations (0.1 µM-1 µM) to the washed resin in 0.5 mL of 0.5X Superdex buffer and incubated for 1 hour at 4°C with constant rotation. The resin was then washed twice as before with 0.5 mL of 0.5X Superdex buffer. Following the wash step, 25 µl of Elution Buffer (100 mM Tris-HCl pH 6.8, 140 mM SDS, 200 mM DTT, 2.3% Bromophenol Blue, 20% glycerol) was added to elute the bound protein. The samples were heated for 10 minutes at 60°C in elution buffer and spun down in a centrifuge to pellet the resin. The supernatant (20 µl) was loaded onto a 15% acrylamide gel and separated by electrophoresis using a Tris-Glycine buffer (10% Tris-Glycine[1.92 M Glycine, 0.25 M Tris], 10% SDS). The proteins were then transferred to an Immobilon-P membrane (Millipore) at 4°C in 1X Tris-Glycine for 1 hour at 44V. The membrane was incubated overnight in 50 mL of 1X TBS/Blotto (100mM Tris-Cl pH 8.0, 1.5M NaCl, 5% nonfat dry milk) with constant shaking. The following day the membrane was incubated in 10mL of TBST/Blotto (100mM Tris-Cl

pH 8.0, 1.5M NaCl, 0.05% Tween 20, 5% nonfat dry milk) and a 1:10000 dilution of an anti-p53 primary antibody (p53 DO1[epitope 11-35] or Pab 1801[epitope 32-79]; Santa Cruz) for 1 hour. Excess primary antibody was removed by three successive 5 minute washes with TBST. The washed membrane was then incubated in 10 mL of TBST/Blotto and a 1:5000 dilution of secondary antibody (goat-antimouse IgG-HRP sc-2055; Santa Cruz) for 30 minutes. The membrane was washed four times with TBST for five minutes to remove the excess secondary antibody. The protein was detected by autoradiography through chemiluminescence using ECL Plus (Amersham). The membrane was Coomassie stained to ensure equal molar concentrations of protein inputs for the GST control and GST-fusion proteins.

Results

The AAD of p53 interacts with transcription factor GATA-1

GATA-1 is critical for regulation of erythroid cell development. The transcription factor PU.1 interacts specifically with the ZBDs of GATA-1, and inhibits its ability to activate transcription [21]. The amino-terminus of PU.1 contains an AAD with similar amino acid composition to the AAD of tumor suppressor p53 and this domain of PU.1 specifically interacts with the ZBDs of GATA-1 [21]. Therefore, we examined whether the AAD of p53 also interacts with the ZBDs GATA-1. [22]. A GST-fusion protein containing, the NZ, the linker region, the CZ and the BA of GATA-1 (GATA-$1_{(199-317)}$) was purified and incubated with increasing concentrations of purified recombinant $p53_{(1-}$

$_{72)}$ in a GST pull-down assay. According to our results, the AAD of p53 interacts specifically with the zinc finger region of GATA-1$_{(199-317)}$.

The AAD of p53 can be further divided into two subdomains, AAD1 (p53$_{(1-40)}$) and AAD2 (p53$_{(40-73)}$). Deletion mutants of the p53 AAD were purified in order to define the minimal region competent for GATA-1 interaction (Figure 4.2) In a GST pull-down assay, a fragment consisting of only AAD1, amino acids 1 to 40, was incubated with GST-GATA-1$_{(199-317)}$ (Figure 4.3). The removal of AAD2 resulted in a loss of interaction suggesting that the region required for binding lies between residues 40 and 73. Since p53$_{(20-73)}$ encompasses a region not present in the p53$_{(1-40)}$ fragment, it could be assumed that its binding affinity would be comparable to that observed with p53$_{(1-72).}$ To evaluate this, the *in vitro* assay was repeated with p53$_{(20-73)}$ since it contains AAD2 and a small portion of AAD1. This experiment revealed that the p53$_{(20-73)}$ construct also was not sufficient for binding GATA-1$_{(199-317)}$ (Figure 4.4). Based on these results, it appears the complete AAD of p53 is required for interaction with GATA-1.

Identification of the minimal p53-interacting region of GATA-1

After establishing that the entire AAD of p53 was required for interaction with GATA-1, we precisely mapped the binding region for p53 on GATA-1. Constructs extending across the ZBDs of GATA-1 were purified to define the minimal region for interaction with p53. These constructs include the NZ plus the linker region (GATA-1$_{(200-251)}$), the linker region, plus the CZ, plus the BA (GATA-1$_{(228-317)}$), and a fragment containing just the CZ and BA (GATA-1$_{(252-317)}$). First, GATA-1$_{(200-251)}$ was incubated with various concentrations of p53$_{(1-72)}$ in a GST pull-down assay. This experiment revealed that the

NZ and linker region of GATA-1 was not sufficient for the interaction with the AAD of p53 (Figure 4.5). The same assay was then repeated with the GATA-$1_{(228-317)}$ fragment. This fragment contains the linker region, the CZ and the BA regions of GATA-1. The incubation of $p53_{(1-72)}$ with this construct resulted in binding comparable to that observed with GATA-$1_{(199-317)}$ (Figure 4.6). Finally, the fragment containing only the CZ and BA of GATA-1 (GATA-$1_{(252-317)}$) was assayed for its ability to bind to $p53_{(1-72)}$. The results demonstrated that GATA-$1_{(252-317)}$ was not sufficient for binding the AAD of p53 (Figure 4.7). Therefore, we propose that the binding interface for the interaction between the AAD of p53 and GATA-1 lies between residues 228 and 317 and requires both the linker region, the CZ and the BA of GATA-1.

The AAD of p53 also interacts with the zinc-finger region of GATA-2

Along with GATA-1, GATA-2 is the other factor that is absolutely required for normal hematopoiesis [23]. The ZBDs of GATA-2 are very homologous to those of GATA-1. We were interested in determining whether the ability of GATA-1 to interact with p53 was conserved throughout the other GATA factors. The ZBD region (NZ, linker, CZ and BA) of GATA-2 (GATA-$2_{(280-412)}$) was purified as a GST-fusion protein for use *in vitro* binding assays. Various concentrations of $p53_{(1-72)}$ were then incubated with GATA-$2_{(280-412)}$ in a GST pull-down assay. Results of this experiment revealed that the AAD of p53 also interacts specifically with GATA-$2_{(280-412)}$ (Figure 4.9). Based upon this result, we believe that p53 has the potential to interact with all GATA factors, given the sequence homology between the ZBDs of all GATA factors.

Discussion

The development of tissue-specific cells is dependent upon the regulation of gene expression by cell-type specific transcription factors. GATA-1 mediates several developmental events in red blood cells and Sertoli cells [8, 14]. The transcriptional activity of GATA-1 in red blood cells is also controlled by a number of other cellular transcription factors. For example, in cooperation with the retinoblastoma protein, PU.1 represses the transcriptional activity of GATA-1 [24]. Interestingly, the AAD of PU.1 has similar amino acid composition to the AAD of p53. In this work we demonstrate that like the AAD of PU.1, the AAD of p53 interacts with the ZBD region of GATA-1. In addition, the AAD of p53 also interacts with the highly homologous zinc finger region of GATA-2 and this suggests that the AAD of p53 is capable of directly binding to all GATA factors.

Although the relationship between p53 and GATA factors has been examined in knockout cells, a direct interaction between the two proteins has not been reported. The AAD of p53 is composed of two subdomains (AAD1 and AAD2) that span the first 72 residues of the protein. To map the minimal binding region required for interaction, deletion mutants of the p53 AAD were examined for their ability to bind GATA-1. Based on our results, it appears that the first 60 amino acids of p53 are required for interaction with GATA-1 (Figure 4.4). This result is similar to what is seen with the PU.1/GATA-1 complex. PU.1 uses both its amino-terminal AAD and its carboxyl-terminal DNA-binding domain for interaction with GATA-1, but the AAD is the only domain required only for inhibition of GATA-1 mediated transcription [21]. Based on

our results, the AAD of p53 is sufficient for interaction with the ZBD region of GATA-1 and this result is similar to the interaction between the AAD of PU.1 and the ZBD of GATA-1.

In addition, we have mapped the minimal region of GATA-1 sufficient for binding to p53. *In vitro* binding studies identified the linker region, the CZ and the BA of GATA-1 as the minimal site sufficient for binding to the AAD of p53 (Figure 4.8). Binding was not observed when assays were performed with the NZ plus the linker region or the fragment containing only the CZ plus BA regions. Although the linker region, CZ and BA are adequate for interaction with p53 *in vitro*, the entire ZBD may be required for full functional activity. As previously stated, other proteins have been shown to interact with the ZBD region of GATA-1 including PU.1, CBP, and FOG [12, 15, 21] .

In addition to our studies with GATA-1, we also determined that p53 interacts with the ZBD region of GATA-2 (Figure 4.9). Evolutionary studies have determined that the six GATA factors found in vertebrates are descended from a common ancestor. Interestingly, the CZ and BA are the only functional domains conserved throughout all members of the GATA family [9]. Therefore, since GATA factors play a role in development this may explain why a regulator of cell-cycle arrest and apoptosis interacts with this region.

The GATA-1/p53 interaction is very similar to the interaction between p53 and human estrogen receptor (ER). ER contains two zinc fingers analogous to the ZBD region of GATA-1 [25]. *In vitro* binding assays have shown the ZBD region of ER also binds the amino-terminal AAD of p53 [26]. Interaction with ER protects p53 from MDM2-

mediated degradation allowing it to accumulate in the nucleus [27]. This inhibition of p53 degradation is essential for p53-induced apoptosis. This mechanism may also occur during the development of hematopoietic cells with GATA-1 stabilizing p53 and preventing it from degradation by MDM2. In fact, it has been shown that p53 is involved in the induction of apoptosis in hematopoietic cell lines [28]. It is also possible that the p53/GATA-1 interaction involves the regulation of transcription. Previous studies have shown that p53 represses the transcriptional activity of ER via direct interaction with its DNA-binding domain [29]. The ZBD region of GATA-1 is also the important for DNA-binding to the target sequence AGATAA. In fact, the amino-terminal AAD of PU.1 is able to alter GATA-1 function by inhibiting GATA-1 binding to DNA [30]. This suggests that by interacting with the DNA-binding domain of these proteins, p53 inhibits their interaction with specific promoter sequences. Presently, it is not clear what role p53 has in regulating GATA-1 activity or vice versa. Further biochemical and structural studies are needed to define the role of this interaction in red blood cell development.

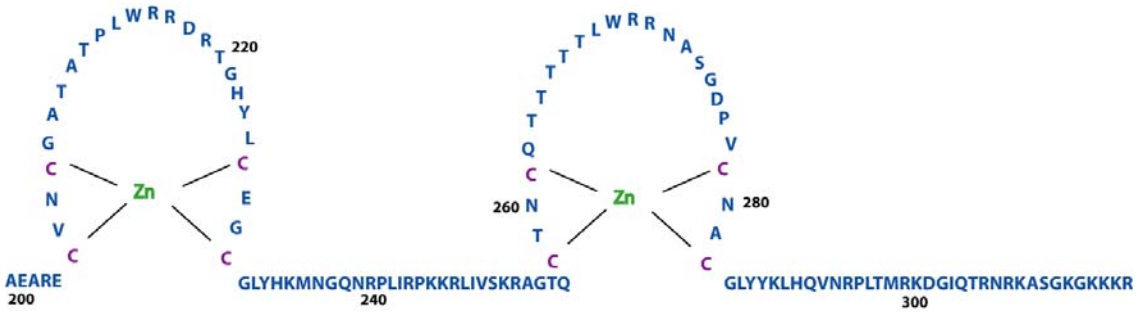


Figure 4.1. Amino acid sequence of the highly conserved ZBDs of GATA-1

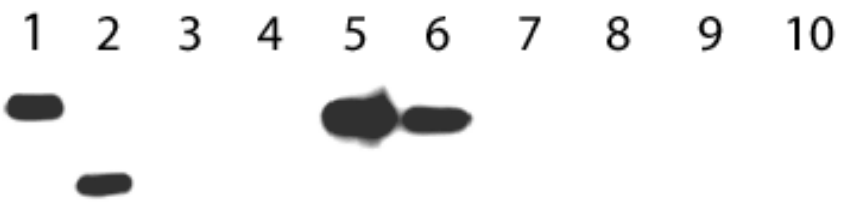


Figure 4.2. *In vitro* binding studies of p53$_{(1-72)}$ and p53$_{(1-40)}$ GATA-1$_{(199-317)}$. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ (lanes 5-7) and p53$_{(1-40)}$ (lanes 8-10) were incubated with 1 μM GST-GATA-1$_{(199-317)}$. In the GST lanes, 1 μM of purified p53$_{(1-72)}$ (lane 3) and p53$_{(1-40)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% p53$_{(1-72)}$ (lane 1) and p53$_{(1-40)}$ (lane 2).

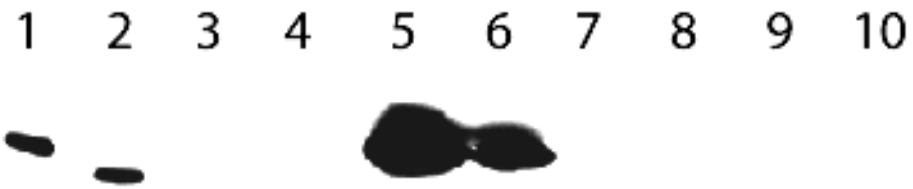


Figure 4.3. *In vitro* binding studies of p53$_{(1-72)}$ and p53$_{(20-73)}$ GATA-1$_{(199-317)}$.. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ (lanes 5-7) and p53$_{(20-73)}$ (lanes 8-10) were incubated with 1 μM GST-GATA-1$_{(199-317)}$. In the GST, lanes 1 μM of purified p53$_{(1-72)}$ (lane 3) and p53$_{(20-73)}$ (lane 4) were incubated with 1 μM GST as a control. The input lanes are 0.5% p53$_{(1-72)}$ (lane 1) and p53$_{(20-73)}$ (lane 2).

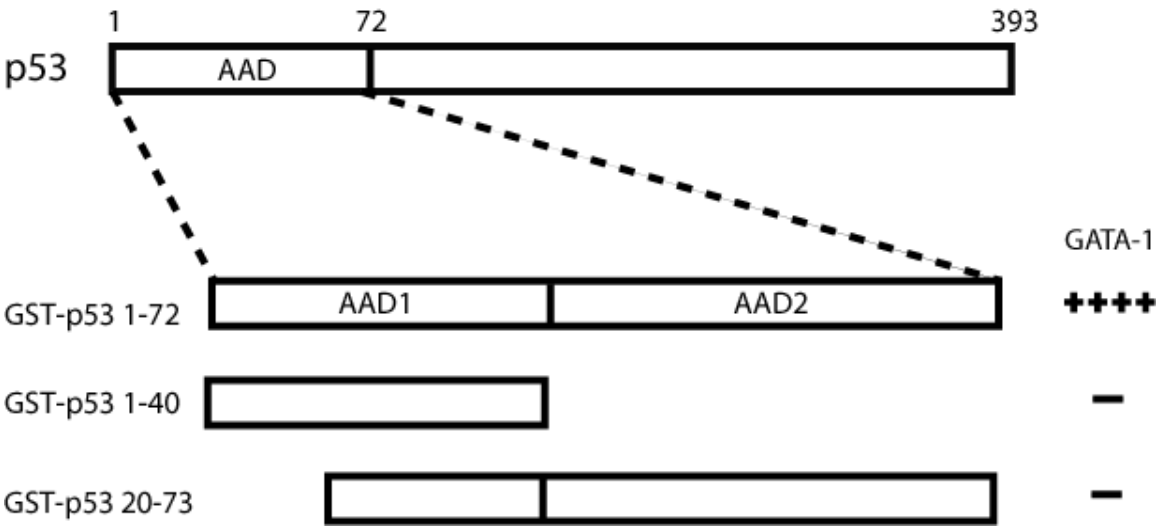


Figure 4.4. The entire AAD of p53 is required to interact with the ZBD region of GATA-1. Deletion mutants of the p53 AAD and a summary of their binding affinity to the ZBD region of GATA-1.

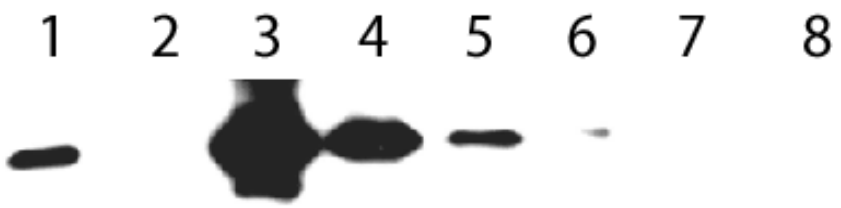


Figure 4.5. *In vitro* binding studies of p53$_{(1-72)}$ with GATA-1$_{(199-317)}$ and GATA-1$_{(200-251)}$. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ was incubated with either 1 μM GST-GATA-1$_{(199-317)}$ (lanes 3-5) or GST-GATA-1$_{(200-251)}$ (lanes 6-8). In the GST lane, 1 μM of purified p53$_{(1-72)}$ were incubated with 1 μM GST as a control (lane 2). The input lane is 0.5% p53$_{(1-72)}$ (lane 1).

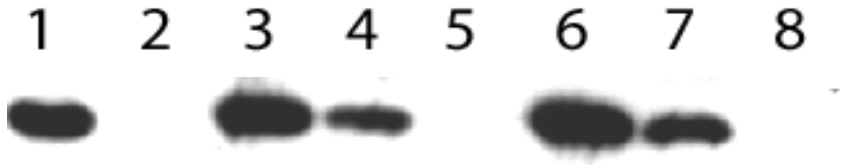


Figure 4.6. *In vitro* binding studies of p53$_{(1-72)}$ with GATA-1$_{(199-317)}$ and GATA-1$_{(228-317)}$.. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ was incubated with either 1 μM GST-GATA-1$_{(199-317)}$ (lanes 3-5) or GST-GATA-1$_{(228-317)}$ (lanes 6-8). In the GST, lane 1 μM of purified p53$_{(1-72)}$ were incubated with 1 μM GST as a control (lane 2). The input lane is 0.5% p53$_{(1-72)}$ (lane 1).

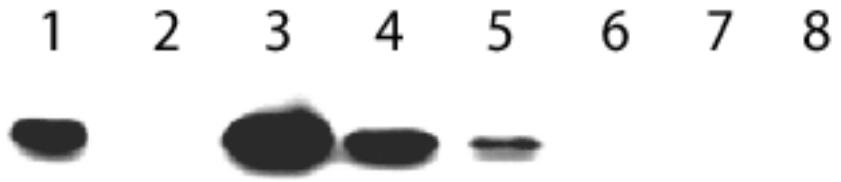


Figure 4.7. *In vitro* binding studies of p53$_{(1-72)}$ with GATA-1$_{(199-317)}$ and GATA-1$_{(252-317)}$. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ was incubated with either 1 μM GST-GATA-1$_{(199-317)}$ (lanes 3-5) or GST-GATA-1$_{(252-317)}$ (lanes 6-8). In the GST lane 1 μM of purified p53$_{(1-72)}$ was incubated with 1 μM GST as a control (lane 2). The input lane is 0.5% p53$_{(1-72)}$ (lane 1).

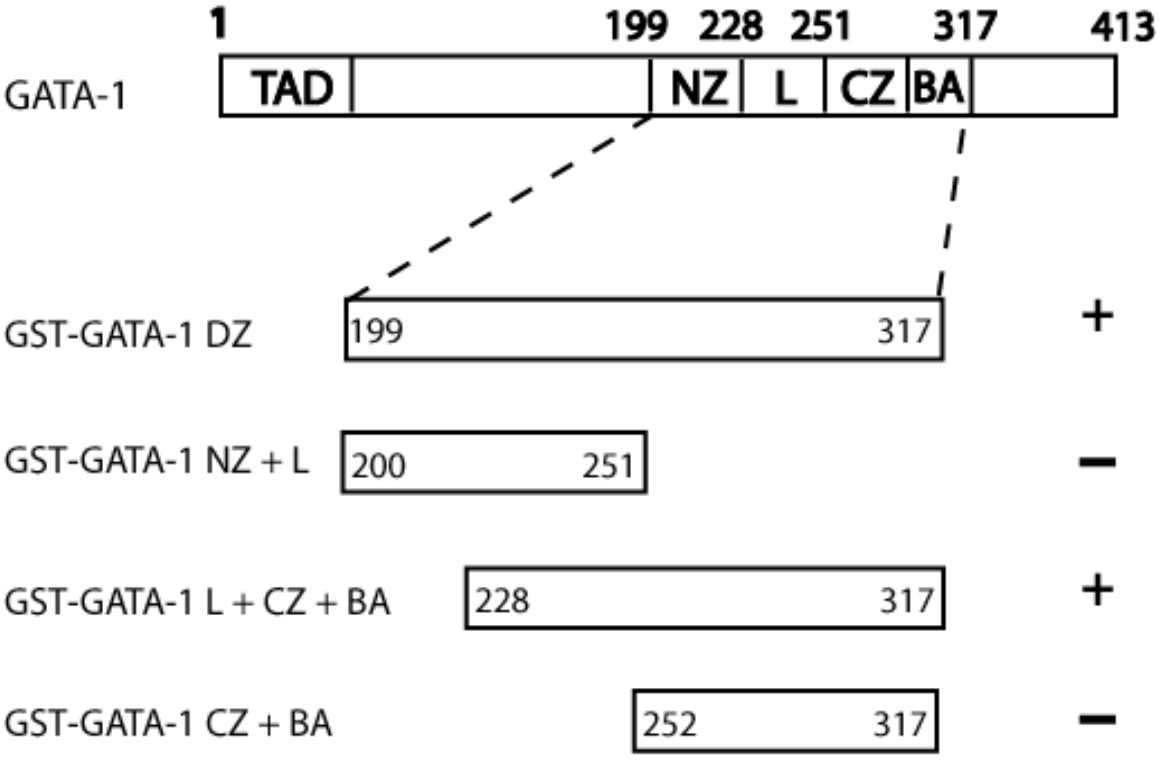


Figure 4.8. Interaction with the AAD of p53 requires the linker region, CZ and BA of GATA-1. Deletion mutants of the ZBD region of GATA-1 and a summary of their binding affinity to the AAD of p53.

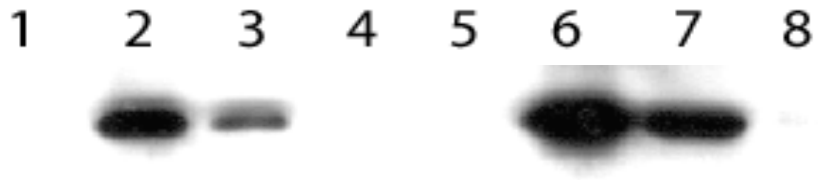


Figure 4.9. *In vitro* binding studies of p53$_{(1-72)}$ with GATA-2$_{(280-412)}$ and GATA-1$_{(199-317)}$. Varying concentrations (1.0, 0.5, 0.1 μM) of p53$_{(1-72)}$ was incubated with either 1 μM GST-GATA-2$_{(280-412)}$ (lanes 3-5) or GST-GATA-1$_{(199-317)}$ (lanes 6-8). In the GST lane, 1 μM of purified p53$_{(1-72)}$ were incubated with 1 μM GST as a control (lane 1). The input lane is 0.5% p53$_{(1-72)}$ (lane 2).

References

1. Evans, T., M. Reitman, and G. Felsenfeld, *An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes.* Proc. Natl. Acad. Sci. USA, 1988. **85**(16): p. 5976-80.

2. Pevny, L., et al., *Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1.* Nature, 1991. **349**(6306): p. 257-60.

3. Tsai, F.Y., et al., *An early haematopoietic defect in mice lacking the transcription factor GATA-2.* Nature, 1994. **371**(6494): p. 221-6.

4. Pandolfi, P.P., et al., *Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis.* Nature Genet, 1995. **11**(1): p. 40-4.

5. Arceci, R.J., et al., *Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart.* Mol. Cell. Biol., 1993. **13**(4): p. 2235-46.

6. Morrisey, E.E., et al., *GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development.* Dev Biol, 1997. **183**(1): p. 21-36.

7. Morrisey, E.E., et al., *GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm.* Dev. Biol., 1996. **177**(1): p. 309-22.

8. Ohneda, K. and M. Yamamoto, *Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage.* Acta Haematol., 2002. **108**(4): p. 237-45.

9. Lowry, J.A. and W.R. Atchley, *Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain.* J. Mol. Evol., 2000. **50**(2): p. 103-15.

10. Morrisey, E.E., et al., *GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily.* J. Biol. Chem., 1997. **272**(13): p. 8515-24.

11. Omichinski, J.G., et al., *NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1.* Science, 1993. **261**(5120): p. 438-46.

12. Blobel, G.A., et al., *CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation.* Proc. Natl. Acad. Sci. USA, 1998. **95**(5): p. 2061-6.

13. Martin, D.I. and S.H. Orkin, *Transcriptional activation and DNA binding by the erythroid factor GF-1/NF-E1/Eryf 1.* Genes & Dev., 1990. **4**(11): p. 1886-98.

14. Yomogida, K., et al., *Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells.* Development, 1994. **120**(7): p. 1759-66.

15. Tsang, A.P., et al., *FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation.* Cell, 1997. **90**(1): p. 109-19.

16. Pevny, L., et al., *Development of hematopoietic cells lacking transcription factor GATA-1.* Development, 1995. **121**(1): p. 163-72.

17. Shivdasani, R.A., et al., *A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development.* EMBO J., 1997. **16**(13): p. 3965-73.

18. Weiss, M.J. and S.H. Orkin, *Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis.* Proc. Natl. Acad. Sci. USA, 1995. **92**(21): p. 9623-7.

19. Tsai, F.Y. and S.H. Orkin, *Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation.* Blood, 1997. **89**(10): p. 3636-43.

20. Moreau-Gachelin, F., A. Tavitian, and P. Tambourin, *Spi-1 is a putative oncogene in virally induced murine erythroleukaemias.* Nature, 1988. **331**(6153): p. 277-80.

21. Rekhtman, N., et al., *Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells.* Genes & Dev., 1999. **13**(11): p. 1398-411.

22. Klemsz, M.J. and R.A. Maki, *Activation of transcription by PU.1 requires both acidic and glutamine domains.* Mol. Cell. Biol., 1996. **16**(1): p. 390-7.

23. Fujiwara, Y., et al., *Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development.* Blood, 2004. **103**(2): p. 583-5.

CHAPTER 5

CONCLUSIONS

Activators affect many facets of eukaryotic transcription including interacting with regulatory elements and recruiting components of the transcriptional machinery and chromatin remodeling complexes. Additionally, activators have been shown to stimulate the rate of initiation and elongation. These functions typically occur through protein/protein interactions between the activator and its target. Activators are able to interact with several cellular factors, thereby affecting many stages of the transcriptional process. We have described the interaction between the activator p53 and three cellular targets TFIIH, CBP, and GATA-1. These interactions could potentially affect various stages of transcription including interaction with target DNA sequences, formation of the pre-initiation complex, and the stimulation of elongation.

Previous studies have shown that the activators p53 and VP16 stimulate the initiation and elongation stages of transcription. This activity correlates with their ability to interact with a subunit of general transcription factor TFIIH, Tfb1 and its human homologue p62. The activation domains of p53 and VP16 are very similar to the amino acid composition of the acid-rich carboxyl-terminal domain of TFIIEα. Similar to p53 and VP16, TFIIEα has also been shown to interact with both the yeast and human Tfb1/p62 subunit of TFIIH. Therefore, given the similarities in their sequence we were interested in determining whether p53, VP16 and TFIIEα share the same binding site of Tfb1/p62. Using a combination of *in vitro* binding studies

and NMR spectroscopy we have shown that the acid-rich domains of p53, VP16, and TFIIEα interact with the amino terminus of Tfb1. Interestingly, each of these targets affects residues in and around the α1 helix of Tfb1. Based on results showing that residues undergoing significant chemical shift changes in the Tfb1/p53, Tfb1/VP16, and Tfb1/TFIIEα complexes are strikingly similar, we feel that p53, VP16, and TFIIEα may compete for the same binding site on Tfb1 *in vivo*. In the cell, each of these components is involved in the regulation of transcription. Therefore, it is very feasible that p53, VP16, and TFIIEα may bind to Tfb1 using a similar mechanism in order to stimulate elongation.

Another cellular target of p53 is the nuclear coactivator cyclic-AMP response element binding protein (CBP) and its yeast homologue p300. p53 has been shown to interact with CBP/p300 through its CH1, KIX, CH3, and IBiD domains. These interactions are important for regulating the degradation, transactivation and post-translational modifications of p53. In this study we have further defined the binding site for both the IBiD and KIX domains within the acidic activation domain (AAD) of p53 using *in vitro* binding assays and NMR spectroscopy. We have determined that the KIX domain interacts with residues in AAD2 (amino acids 40-73) of p53. The first subdomain of the AAD of p53 (amino acids 1-40) is not sufficient for interaction with the KIX domain, but contains residues required for binding. The interaction with IBiD domain however, requires amino acids 25 to 65, however does not need residues present in AAD1. Therefore, it appears that although the KIX and IBiD domains interact with the AAD of p53, each binds to a different site. It has been shown that the interaction between p53 and the IBiD domain of CBP stimulates the rate of transcription. There is also evidence that the cellular

protein Tax may compete with p53 for interaction with the KIX and IBiD domains of CBP. This competition also inhibits p53-mediated transcription. Based on this data it appears that Tax affects the ability of p53 to activate transcription by preventing it from interacting with these domains.

Lastly, we examined the interaction between p53 and members of the GATA transcription factor family. The first factor of this family to be cloned and characterized, GATA-1, has been shown to interact with another transcription factor, PU.1, which contains an AAD with similar amino acid composition to the AAD of p53. Although PU.1 has the ability to interact with GATA-1 using its amino-terminal AAD and its carboxyl-terminal DNA-binding domain (DBD), the AAD is the only domain required for inhibition of GATA-1 mediated transcription. Therefore, we were interested in whether the AAD of p53 interacts with GATA-1 as well. Using *in vitro* binding assays we found that similar to the AAD of PU.1, the AAD of p53 interacts with the zinc-binding domain (ZBD) of GATA-1. This domain is highly conserved throughout members of the GATA transcription factor family. We performed an additional *in vitro* binding assay which showed p53 also interacts with the ZBD of GATA-2. A possible implication of this interaction on transcription in the cell is preventing GATA-1 from binding to DNA. By interacting with the DNA-binding domain of GATA-1, p53 inhibits its interaction with specific promoter sequences and represses GATA-1 mediated transcription. Human estrogen receptor (ER) contains two zinc fingers analogous to the ZBD of GATA-1 and interacts with the AAD of p53 through this region. Previous studies have shown that p53 represses the transcriptional activity of ER by interacting with its DBD and preventing it from binding to DNA.