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Since this grant was awarded (7/1/96), we have constructed a set of deletions to disrupt the domains of p53 responsible for nuclear localization (aa 316 to 322), tetramerization (aa 325 to 356) and the regulation of the DNA binding activity of p53 (aa 363 to 393). Furthermore, the ability of these mutants to interact with c-Abl was also tested using a GST pull-down assay. Our results show that deletion of last 30 amino acids in p53 severely disrupted its ability to bind to c-Abl and deletion of the tetramerization domain also greatly reduced the binding to c-Abl. Based on these results, we propose a model in which c-Abl interacts with the regulatory domain (aa 363 to 393) in p53 to diminish its negative regulatory effect and to enhance the DNA binding activity of p53. This interaction, however, requires the tetrameric conformation of the protein. To test this requirement, ability of a mutant p53 (341K344E348E355K, tetramerization impair) to interact with c-Abl was investigated. Our results show this mutant is defective in c-Abl interaction. We are currently examining the effect of c-Abl on DNA binding activity of p53.

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FOREWORD

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Introduction:

The loss of cell growth regulation is a hallmark of cancer. To achieve our goal of designing therapies for cancer, we must understand how cancer proteins affect cell growth. The aim of this proposal is to address this question for the cancer related proteins, p53 and c-Abl. Our previous work demonstrated that c-Abl requires p53 for growth suppression. In studying the mechanism of this effect, we find that c-Abl can enhance the transcriptional activity of p53 *in vivo*. This enhancement requires a domain in c-Abl that mediates binding to p53 (Goga, Liu et al, 1995). Because we are unable to observe a direct phosphorylation of p53 by c-Abl, we hypothesize that c-Abl may function through the following possible mechanisms to activate p53-dependent transcription: 1) c-Abl enhances the DNA-binding activity of p53; 2) c-Abl brings other regulators to the promoter; 3) c-Abl phosphorylates general transcription machinery, which in turn allows p53 to function. In keeping with this trend, we have proposed:

1. To define the domains on p53 required for c-Abl binding and correlate ability of p53 to interact with c-Abl with its ability to respond to c-Abl's stimulation

2. To examine the effect of c-Abl on the DNA-binding activity of p53

3. To characterize the effect of c-Abl on p53-dependent transcription *in vitro* and on the interaction of p53 with its functional targets (TBP and TAF250)

4. To determine the effect of Gal4-Abl on transcription from a promoter containing Gal4 binding sites

5. To examine whether general transcription factors are phosphorylated by c-Abl and the role of phosphorylation in transcription regulation

Background and Previous Work

p53 is a very important tumor suppressor gene (Finlay et al, 1989). Alteration or loss of p53 is associated with a wide variety of human tumor cells (Hollstein et al, 1991; Lane and Benchimol, 1990; Levine et al, 1991). p53 is believed to act as a G1 checkpoint control by causing growth arrest and inducing apoptosis in cases where DNA damage is too severe to be repaired (Yonish-Rouach et al. 1991). p53 has been identified as a sequence-specific DNA-binding transcription factor (Bargonetti et al, 1991; Kern et al, 1992; Farmer et al, 1992). Considerable evidence indicates that transcriptional activity of p53 is critical for its function as a tumor suppressor (Vogelstein and Kinzler 1992; Yew and Berk 1992). In response to double-strand DNA breaks (Nelson and Kastan, 1994), p53 is thought to activate the expression of proteins that arrest cells at the G1 to S phase transition (Kastan et al, 1992; Lu and Lane, 1993). Among the genes induced by p53 are p21 (El-Deiry et al. 1993), which encodes a protein that binds and inhibits all currently known Cdk-cyclin protein kinases required for the G1 to S phase transition (Harper et al, 1993). Similarly,

induction of apoptosis by p53 is thought to involve activation of the Bax gene which encodes an accelerator of apoptosis (Miyashita and Reed, 1995) and the Fas/APO gene which encodes a protein known to trigger apoptosis (Owen-Schaub et al, 1995). Therefore, inactivation of p53 may predispose to oncogenic transformation and tumor progression by disrupting a normal cell cycle check point required for repair of DNA damage before entry into S-phase and by preventing apoptosis in response to DNA damage.

Based on the G1 arrest phenotype of p53, we reasoned that p53 transcriptional activity must be affected by the cell cycle proteins which regulate G1. c-Abl has been reported to be a growth suppressor and overexpression of c-Abl leads to G1 growth arrest in fibroblasts (Sawyers et al, 1994). The c-Abl protein is a predominantly nuclear tyrosine kinase. The kinase activity of c-Abl is tightly regulated *in vivo*, possibly by binding to unidentified inhibitory proteins (Pendergast et al, 1991), and is required for c-Abl to suppress growth (Sawyers et al, 1994). Links between the c-Abl proto-oncogene and cell cycle suggest that c-Abl normally acts as a negative regulator of cell growth and that it may function through p53. The availability of mouse fibroblasts containing disruptions of the Rb (a very important tumor suppressor gene) or p53 genes allowed us to genetically test this possibility. Our results show that c-Abl requires p53 but not Rb to suppress growth. In addition, we also find that c-Abl binds to p53 *in vitro* and enhances the ability of p53 to activate transcription from a promoter containing a p53 DNA binding site in a transient transfection assay. Deletion of the p53 binding domain in c-Abl (ΔProl, a deletion of proline rich domain, aa 793-1044) impairs the ability of c-Abl to stimulate p53 transcriptional activity and to suppress growth (Goga, Liu et al, 1995). These results suggest that the regulation of p53 transcription is very important in negative growth control by c-Abl. Therefore, a detailed understanding of how c-Abl stimulates p53-dependent transcription may allow the rational design of therapies which can reactivate the Abl-p53 pathway in tumor cells, resulting in cell cycle arrest and apoptosis of tumor cells.

Experimental Methods and Procedures

Construction of mutants

p53ΔC292 mutant was constructed by PCR amplification of amino acid residues 1 to 292 and 1 to 363 of p53 from pcDNA-p53 (Liu et al. 1993), using primers that introduce a BamHI at 5' end and an EcoRI at 3' end. The amplified DNA fragments were then cloned between the BamHI and EcoRI sites of pcDNAI/Amp (Invitrogen). p53ΔC363 was constructed by PCR amplification of amino acid residues 1 to 363 of p53, using primers that introduce a HindIII at 5' end and an EcoRI at 3' end. The amplified DNA fragments were then cloned in the HindIII and EcoRI sites of pcDNAI/Amp. Deletion mutants p53Δ316-322 and p53Δ325-356 were constructed by inverse PCR from pcDNA-p53 with the region to be deleted bracketed between two "back-to back" primers. After amplification by PCR, the fragments were phosphorylated and ligated into circular plasmids. Mutant p53 (341K344E348E355K)

was constructed in pcDNA-p53 by inverse PCR with two "back-to-back" primers which introduce mismatches at corresponding positions. All the mutants were confirmed with DNA sequencing.

p53 and c-Abl Binding Experiments

The GST-Abl fusion protein was expressed and purified as previously described (Gaga, Liu et al, 1995). Wild type and mutant p53 RNAs were transcribed from T7 promoter according to conditions recommended by the manufaturer (Promega). The RNAs were then translated in vitro for 1.5 hrs. at 30°C in rabbit reticulocyte lysate in the presence of ^{35}S-methionine (10 mCi/ml) in a condition recommended by Promega. Radiolabeled in vitro translated proteins were diluted 100-fold with incubation buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol and ethidium bromide 50 μg/ml) and incubated with 1 μg GST or GST-Abl fusion protein bound to Glutathione-Sepharose beads at room temperature for 60 minutes with constant mixing. After incubation, the beads were washed three times with incubation buffer without ethidium bromide. The beads were then boiled in 2X Laemli buffer and the bound proteins were analyzed by SDS-PAGE. ^{35}S-methionine labeled proteins were visualized by autoradiography.

Results and Discussion

c-terminal region is required for c-Abl interaction

In our previous study (Goga, Liu et al, 1995), we have established an interaction between p53 and c-Abl. To study the effect of this interaction on p53-dependent transcription, we have mapped c-Abl interaction domain on p53. This was done by using a GST pull-down assay. In these experiments, GST and GST-Abl were expressed in bacteria and immobilized to GST beads as described by Liu et al (1993). The p53 proteins were ^{35}S-labeled and incubated with immobilized GST beads. After incubation, the beads were washed and the proteins retained on the beads were assayed by SDS-PAGE. Results of representative GST pull-down experiments are shown in Fig. 1. Deletion of the p53 N-terminal 92 and 160 amino acids, Δ92 and Δ160, had no effect on c-Abl binding. In contrast, deletion of a C-terminal region of p53, ΔC292, abolished binding to c-Abl (Fig. 1). Therefore residues between 292 and 393 in p53 are required for the p53-Abl interaction.

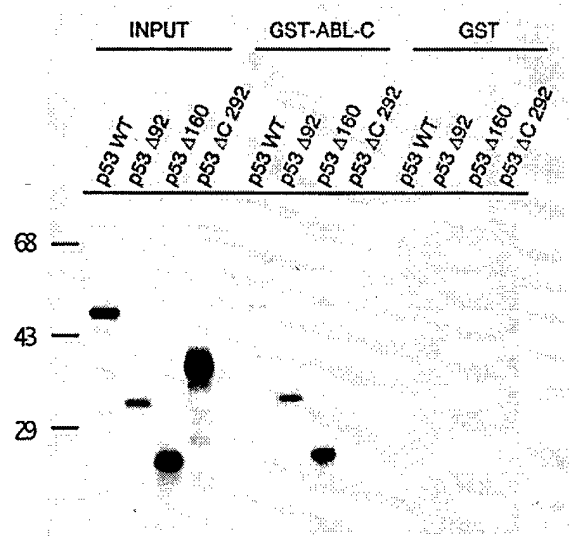

Figure 1. c-Abl binds the carboxyl-terminal domain of p53. p53 deletion mutants were translated *in vitro* and tested for binding to GST-Abl. Input for each of the *in vitro* translated p53 proteins represents 10% of p53 protein used in the binding reactions. Binding of p53 proteins to Abl was measured by incubation with 1 μg of immobilized GST-Abl protein, washing, SDS-PAGE and autoradiography of protein retained on the beads. Right panel shows binding of p53 proteins to GST protein alone immobilized on beads.

c-Abl interacts with c-terminal 30 amino acids of p53 and this interaction requires p53 in a tetrameric conformation

The c-terminal region (293 to 393) can be divided into three functional domains: an extreme carboxyl end (30 residues) which inhibites the ability of p53 to bind specifically to DNA (Hupp et al, 1992), a tetramerization domain (325-356) and a nuclear localization domain (319-323). In order to further characterize the p53-cAbl interaction, we constructed a set of deletions to disrupt the domains of p53 responsible for nuclear localization (aa 316 to 322), tetramerization (aa 325 to 356) and the regulation of the DNA binding activity of p53 (aa 363 to 393); as diagrammed in Fig. 2.

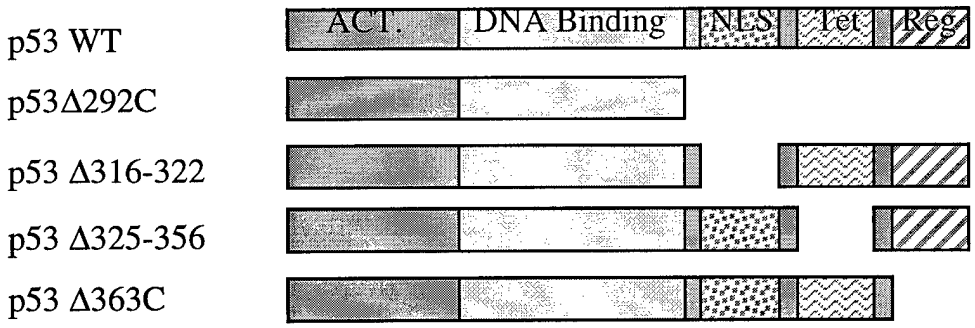

Figure 2. Schematic Diagram of p53 deletions. Act: activation domain; NLS: nuclear localization signal; Tet: tetramerization domain and Reg: a region which can regulates DNA-binding activity of p53.

We then tested the ability of these mutants to interact with c-Abl using a GST pull-down assay in a condition described previously. c-Abl binds to the NLS mutant Δ316-322 as well as wild type (Fig. 3). Deletion of last 30 amino acids in p53, ΔC363, however, severely disrupted its ability to bind to c-Abl. This region has been previously identified as a regulatory domain to inhibit the DNA binding activity of p53.

Furthermore, deletion of the tetramerization domain, Δ325-356, also greatly reduced the binding to c-Abl. Based on these results, we proposed a model in which c-Abl interacts with the regulatory domain (aa 363 to 393) of p53 to diminish its negative regulatory effect on DNA binding. This interaction, however, requires the tetrameric conformation of the protein.

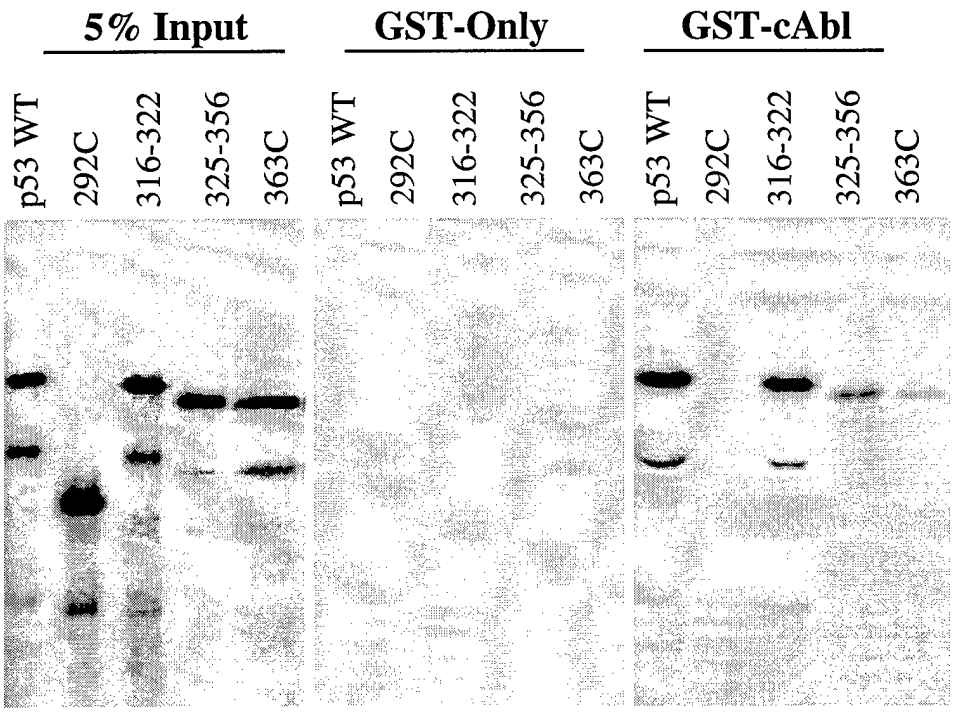

Figure 3. Localization of Binding Domain of c-Abl on the p53 C-terminus. Radiolabeled p53 mutants were produced by *in vitro* translation and incubated with GST-Abl. After washing, proteins were denatured and subjected to SDS-PAGE. Deletion of aa 363 to 393 in p53 (the region which has been previously identified as a negative regulatory domain) severely disrupted its ability to bind to c-Abl. Deletion of the tetramerization domain also greatly reduced the binding to c-Abl.

To test this requirement for a tetrameric conformation, we constructed a tetramerization impair mutant, 341K344E348E355K, which contains four mutanted residues at positions 341, 344, 348 and 355 as described by Sturzbecher et al. 1992. The ability of the mutant to interact with c-Abl was examinated using a GST pull-down assay. As shown in Fig. 4, this mutant continues to fail to bind to c-Abl like Δ325-356, demonstrating the requirement of the tetrameric conformation for c-Abl interaction.

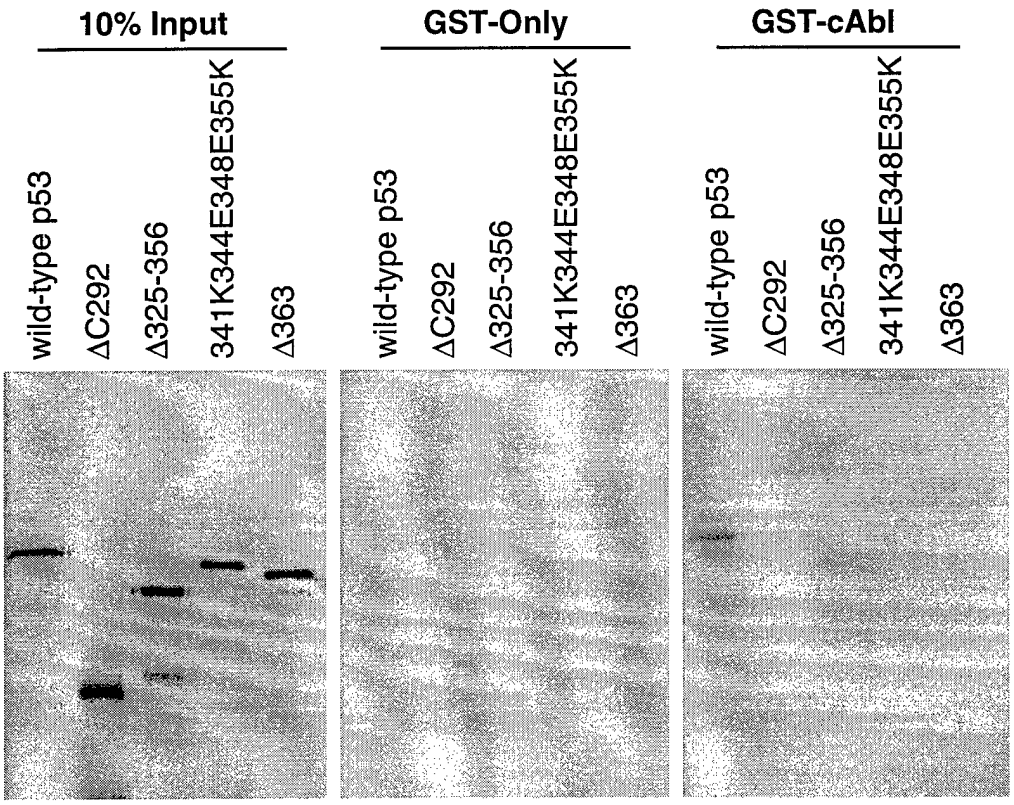

Figure 4. Tetrameric conformation of p53 is reguired for c-Abl interaction. Radiolabeled p53 constructs were incubated with GST (middle panel) or GST-Abl (right panel). Proteins retained on the GST beads were subjected to SDS-PAGE. 341K344E348E355K disrupted its ability to bind to c-Abl.

The finding that the negative regulatory domain interacts with c-Abl may provide important clues about the regulation of p53. It has been suggested by David Lane's group that peptides designed to interact with this region can be used to reactivate p53 pathway in tumor cells to cause cell cycle arrest (Hupp et al. 1995). In spite of the significance of this region, however, the cellular proteins which interact with it were not identified. The interaction of c-Abl with the negative regulatory region provides evidence for the presence of such cellular proteins. In response to different signals, these cellular proteins may differentially regulate the DNA binding activity of p53 to cause growth arrest. Based on this recent result, we are currently testing our model in which c-Abl interacts with the negative regulatory domain to enhance the DNA binding activity of p53.

Recommendation in Relation to the Statement of Work

Proposed research has been complished according to SOW.

Conclusions

We have demonstrated an interaction between c-Abl and p53 c-terminal regulatory region. Based on this result, we propose a model in which c-Abl interacts with the negative regulatory domain to enhance the DNA binding activity of p53. We are currently in a process to test our model.

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