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# Two cellular proteins that bind to wild-type but not mutant p53

(tumor suppressor/DNA-binding protein/protein–protein interaction/two-hybrid system)

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**ABSTRACT** **p53 is a tumor-suppressor protein that can activate and repress transcription. Using the yeast two-hybrid system, we identified two previously uncharacterized human proteins, designated 53BP1 and 53BP2, that bind to p53. 53BP1 shows no significant homology to proteins in available databases, whereas 53BP2 contains two adjacent ankyrin repeats and a Src homology 3 domain. *In vitro* binding analyses indicate that both of these proteins bind to the central domain of p53 (residues 80–320) required for site-specific DNA binding. Consistent with this finding, p53 cannot bind simultaneously to 53BP1 or 53BP2 and to a DNA fragment containing a consensus p53 binding site. Unlike other cellular proteins whose binding to p53 has been characterized, both 53BP1 and 53BP2 bind to the wild-type but not to two mutant p53 proteins identified in human tumors, suggesting that binding is dependent on p53 conformation. The characteristics of these interactions argue that 53BP1 and 53BP2 are involved in some aspect of p53-mediated tumor suppression.**

The p53 protein is the product of a tumor-suppressor gene, with mutation of this gene being the most common genetic change in human cancer (reviewed in ref. 1). Introduction of the wild-type p53 gene (human gene symbol, *TP53*) into cells leads to growth arrest, and DNA damage leads to increases in the level of p53, which suggests that the protein acts as a checkpoint control to regulate entry from $G_1$ phase into S phase (1). At least one mechanism by which the p53 protein acts is the regulation of transcription, as p53 binds to DNA in a site-specific manner, activates transcription of genes containing these specific binding sites, and represses transcription of several genes containing no known binding sites (1). A key aspect to understanding p53 function is the identification and analysis of proteins that interact with it. For example, proteins encoded by the DNA tumor viruses simian virus 40, adenovirus, and human papilloma virus bind to and target p53 for inactivation (1). The cellular proteins MDM2 (2, 3), TATA-binding protein (TBP) (4, 5), and replication factor A (RPA) (6, 7) bind to the transcriptional activation domain of p53, and these interactions modulate the activity of p53 and its associated proteins.

We have used the yeast two-hybrid system (8, 9) to identify two additional proteins of human origin that bind to p53. These two proteins bind to wild-type p53 but fail to bind to mutant p53, suggesting that they may be involved in the ability of wild-type p53 to suppress transformation.§

## MATERIALS AND METHODS

**DNA Manipulations.** The DNA-binding-domain vector was pGBT9 (10) and the plasmid encoding the DNA-binding domain–murine p53 hybrid was pVA3 (11). The activation-domain library constructed by using cDNA from an Epstein–Barr virus-transformed human cell line was as described (12). The 53BP1 hybrid with the Gal4 DNA-binding domain was constructed by ligating the *Bgl* II insert from pSE1107A70, which encodes the largest 53BP1 insert from the library search, into pGBT9. The 53BP2 hybrid with the Gal4 DNA-binding domain was constructed by first treating pSE1107B67, encoding the second-smallest 53BP2 insert, with BAL-31 nuclease to create a DNA fragment encoding the C-terminal 228 aa and ligating this fragment into pGBT9. The activation-domain vector was pSE1107 (12), and the activation domain–p53 hybrid containing aa 4–393 of human p53 was described (11). The mutant p53 hybrids were constructed by replacing the *NcoI–Stu* I fragment of the wild type-human p53 gene with the equivalent fragment from p53175REP1 (13) for the R175H mutant, and the equivalent fragment from pR4-2 (14) for the R273H mutant. The glutathione *S*-transferase (GST)–53BP1 fusion was generated by digesting pSE1107A16, from the library search, with *Bgl* II and ligating the insert fragment to *Bam*HI-digested pGEX-3X (Pharmacia). GST–53BP2 was generated by digesting a BAL-31-deleted derivative of pSE1107B67 with *Bgl* II and ligating the insert fragment to pGEX-3X.

Initial DNA sequencing of library plasmids used a primer that anneals to the sequence encoding the Gal4 activation domain (11). For complete sequences of the inserts, the library plasmids were digested with *Bgl* II to release the insert, which was ligated to *Bam*HI-digested pBluescript SK II (Stratagene). The resulting plasmids were subjected to exonuclease III digestion and sequence analysis using Sequenase (United States Biochemical). The predicted protein sequences were compared against the nonredundant protein database of the National Center for Biotechnology Information by using the program BLAST (15).

**Yeast Methods.** The yeast strain for the library search was Y153 (12) and for β-galactosidase assays was SFY526 (16). Y153 was simultaneously transformed with the p53 hybrid and the activation-domain cDNA library as described (11). Plasmids that produced a positive signal were tested for specificity by using a Gal4-(1–147)–lamin hybrid (16), and by introducing the plasmid encoding the p53 hybrid and the library plasmid into strain SFY527 carrying a different Gal4-regulated reporter gene (16). β-Galactosidase activity was determined (11) with chlorophenol red β-D-galactopyranoside as substrate.

**RNA Analysis.** A human tissue Northern blot was obtained from Clontech. The probes were a 1.3-kb fragment of 53BP1 cDNA (nt 1408–2713) and a 1.3-kb fragment of 53BP2 cDNA (nt 456–1723) and the blot was probed according to the manufacturer's recommendations.

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Abbreviations: CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; GST, glutathione *S*-transferase; PMSF, phenylmethanesulfonyl fluoride; RPA, replication protein A; SH3, Src homology 3; TBP, TATA-binding protein.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U09477 and U09582).

***In Vitro* Binding.** GST, GST–53BP1, and GST–53BP2 were purified from *Escherichia coli* as follows. Cells were pelleted, washed, and lysed in a French pressure cell with 50 mM Tris·HCl, pH 8.0/25% sucrose/4 mM 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) 0.2 mM phenylmethanesulfonyl fluoride (PMSF)/1 mM benzamidine with leupeptin (2 μg/ml) and pepstatin (1 μg/ml). The lysate was centrifuged at 10,000 rpm (Sorvall SS 34 rotor) for 20 min, and the supernatant was centrifuged at 60,000 rpm for 30 min in a Beckman TL100 ultracentrifuge. $MgCl_2$ (10 mM) and DNase I (200 μg/ml) were added to the resultant supernatant before it was passed through a Sepharose CL-4B (Sigma) column in GEX-C buffer [20 mM Hepes, pH 7.0/100 mM NaCl/1 mM dithiothreitol/10% glycerol (vol/vol)/0.4 mM CHAPSO/0.2 mM PMSF, with leupeptin (2 μg/ml) and pepstatin (1 μg/ml)]; the flowthrough was bound to glutathione-agarose (Sigma) in this same buffer plus 1.2 mM EDTA. Full-length murine p53 was purified from a baculovirus/Sf9 insect cell expression system and a fragment of murine p53 (aa 80–320) was purified from *E. coli* as described (17). The GST fusion bound to the agarose beads and p53 were incubated for 2 hr at 4°C, the beads were pelleted for 2 min at high speed in a microcentrifuge, and the supernatant was recovered as the unbound fraction. The pellets were washed three times in 200 μl of GEX-C buffer and the proteins bound to the washed beads were eluted three times by incubation for 10 min with 100 μl of 40 mM glutathione in the same buffer (final pH of 7.5) and centrifugation conditions as above. Eluates were pooled as the bound fraction. Unbound and bound fractions were fractionated by SDS/10% PAGE, transferred to nitrocellulose, and probed with p53-specific antibody PAb240 (Oncogene Science). Detection was by goat anti-mouse IgG conjugated to alkaline phosphatase as described by GIBCO/BRL. The amount of bound and unbound p53 was determined by a Bio-Rad imaging densitometer.

For assaying whether p53 bound to 53BP1 or 53BP2 could bind to DNA containing a p53-binding site, the histidine-tagged p53 fragment (aa 80–320) was incubated for 1 hr at 4°C in 20 mM Tris·HCl, pH 7.4/50 mM NaCl/1.5 mM $MgCl_2$/10% glycerol/0.01% Nonidet P-40 with double-stranded oligonucleotides having the sequences 5′-CCGGAGACATGCCTAGACATGCCT-3′ and 5′-CCGGAGGCATGTCTAGGCATGTCT-3′. The oligonucleotides were annealed and the 4-base overhangs were filled in with DNA polymerase I and $^{32}P$-labeled dNTPs to yield a 28-bp fragment. Ni-NTA agarose beads (Qiagen, Chatsworth, CA) or glutathione-agarose beads bound to the 53BP1 or 53BP2 fusions or to GST were added to the p53/DNA mixture and incubated for 2 hr at 4°C. Beads were pelleted and washed four times in 200 μl of 20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.4 mM CHAPSO/0.2 mM PMSF. p53 was eluted from the Ni-NTA beads with 300 mM imidazole in 100 mM Tris·HCl, pH 7.0/150 mM NaCl and the GST and GST fusions were eluted from the glutathione-agarose beads with 40 mM glutathione in the GEX-C buffer plus 1.2 mM EDTA.

## RESULTS

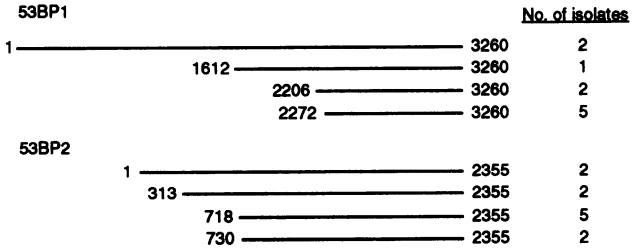
**Two-Hybrid Screen.** The wild-type murine p53 (aa 73–390) was fused to the Gal4 DNA-binding domain (aa 1–147). The p53 domain in this hybrid protein is incapable of activating transcription of a gene containing Gal4 binding sites in yeast, yet it can interact with simian virus 40 large tumor antigen fused to the Gal4 activation domain (aa 768–881) to result in transcriptional activity (11). The p53 hybrid was used as a target in a two-hybrid screen (9) for interacting proteins encoded by a library of cDNAs generated from a transformed human B-cell line and fused to DNA sequences encoding the Gal4 activation domain (12). This screen would avoid detecting proteins that bind to the p53 activation domain and would detect only those proteins that bind to the conformationally sensitive central domain of p53 or the C-terminal oligomerization domain. We plated 700,000 yeast transformants in the absence of histidine to select for those that could express a *GAL1–HIS3* gene, which is regulated by Gal4 binding sites. $His^+$ transformants were then screened for the expression of a second reporter gene, *GAL1–lacZ*. Plasmids from the library that activated both genes were tested for specificity by pairing them with a plasmid encoding a hybrid of the Gal4 DNA-binding domain with a protein unrelated to p53. A total of 57 library plasmids whose activity was specific to p53 were further characterized by DNA sequence analysis. Of these, 36 encoded all or a portion of p53 (11). The remaining 21 encoded one of two human proteins that we designate 53BP1 and 53BP2 (for p53-binding proteins 1 and 2). 53BP1 was isolated 10 times, with these plasmids containing four different classes of inserts ranging from 989 to 3260 bp in length (Fig. 1*A*). 53BP2 was isolated 11 times, also as four different inserts ranging from 1626 to 2355 bp (Fig. 1*A*). All 21 plasmids had the reading frame of the insert in frame with the Gal4 activation domain and contained a portion of the 3′ end of the cDNA extending into the poly(A) tail.

The deduced protein sequence of the largest clone of 53BP1 contains 1027 aa (Fig. 1*B*) and displays no significant homology to proteins in available databases. The smallest clone contains the C-terminal 270 residues of this protein, indicating that this small fragment is sufficient for p53 binding. The largest 53BP2 clone encodes 529 aa (Fig. 1*C*). Although 53BP2 does not have extensive homology to other proteins, it contains two adjacent copies of the ankyrin repeat and a single SH3 domain (Fig. 1*D*). The ankyrin repeat of 33 aa has been found in a diverse set of proteins that function in the nucleus, cytosol, mitochondria, and plasma membrane, and it may mediate protein–protein interactions (24). The SH3 domain of ≈50 aa is found in a number of cytoplasmic proteins that are implicated in signal transduction pathways; this domain binds to certain proline-rich sequences (25). The smallest fragment of 53BP2 that can bind to p53 consists of the C-terminal 228 aa, which include the ankyrin repeats and the SH3 domain.

Since even the largest inserts in the activation domain plasmid obtained from the library search were likely to be only partial cDNAs, we used these inserts to probe Northern blots to identify the corresponding full-length transcripts. The 53BP1 probe detected two transcripts of approximately 11 and 6.8 kb, and the 53BP2 probe detected two transcripts of 4.4 and 2.2 kb (Fig. 2). Absolute levels of these transcripts varied in the different tissues assayed; for example, expression of 53BP1 was not observed in lung and liver, and expression of 53BP2 was markedly reduced in heart and brain. Additionally, the relative abundance of the two 53BP2-related transcripts varied from predominantly the 4.4-kb species (e.g., in skeletal muscle) to predominantly the 2.2-kb species (e.g., in placenta).

**Specificity of 53BP1 and 53BP2 Binding.** We used two-hybrid assays to assess the ability of 53BP1 and 53BP2 to bind to the human wild-type p53 protein and to two mutant p53 proteins identified in tumors. For these experiments, the 1027 aa of the largest 53BP1 insert and the C-terminal 228 residues of 53BP2 were fused to the Gal4 DNA-binding domain. This 53BP1 hybrid did not activate transcription in yeast (Table 1). The 53BP2 hybrid activated slightly (Table 1), although much less than when the largest 53BP2 isolate, 529 aa, was fused to the Gal4 DNA-binding domain (data not shown). A plasmid encoding either the 53BP1 hybrid or the 53BP2 hybrid was introduced into yeast along with one encoding the human p53 protein (aa 4–393) fused to the Gal4 activation domain. The p53 domain was derived either from the wild-type gene, an R175H ($Arg^{175} \rightarrow$ His), or an R273H mutant. Only the wild-type p53 protein was capable of interacting with either

**A**




**B**

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PESTIATSDV MSESMVETHD PILGSGKGDS GAAPDVDDKL CLRMKLVSPE TEASEESLQF NLEKPATGER   70
KNGSTAVAES VASPQKTMSV LSCICEARQE NEARSEDPPT TPIRGNLLHF PSSQGEEEKE KLEGDHTIRQ  140
SQQPMKPISP VKDPVSPASQ KMVIQGPSSP QGEAMVTDVL EDQKEGRSTN KENPSKALIE RPSQNNIGIQ  210
TMECSLRVPE TVSAATQTIK NVCEQGTSTV DQNFGKQDAT VQTERGSGEK PVSAPGDDTE SLHSQGEEEF  280
DMPQPPHGHV LHRHMRTIRE VRTLVTRVIT DVYYVDGTEV ERKVTEETEE PIVECQECET EVSPSQTGGS  350
SGDLGDISSF SSKASSLHRT SSGTSLSAMH SSGSSGKGAG PLRGKTSGTE PADFALPSSR GGPGKLSPRK  420
GVSQTGTPVC EEDGDAGLGI RQGGKAPVTP RGRGRRGRPP SRTTGTRETA VPGPLGIEDI SPNLSPDDKS  490
FSRVVPRVPD STRRTDVGAG ALRRSDSPEI PFQAAAGPSD GLDASSPGNS FVGLRVVAKW SSNGYFYSGK  560
ITRDVGAGKY KLLFDDGYEC DVLGKDILLC DPIPLDTEVT ALSEDEYFSA GVVKGHRKES GELYYSIEKE  630
GQRKWYKRMA VILSLEQGNR LREQYGLGPY EAVTPLTKAA DISLDNLVEG KRKRRSNVSS PATPTASSSS  700
STTPTRKITE SPRASMGVLS GKRKLITSEE ERSPAKRGRK SATVKPGAVG AGEFVSPCES GDNTGEPSAL  770
EEQRGPLPLN KTLFLGYAFL LTMATTSDKL ASRSKLPDGP TGSSEEEEEF LEIPPFNKQY TESQLRAGAG  840
YILEDFNEAQ CNTAYQCLLI ADQHCRTRKY FLCLASGIPC VSHVWVHDSC HANQLQNYRN YLLPAGYSLE  910
EQRILDWQPR ENPFQNLKVL LVSDQQQNFL ELWSEILMTG GAASVKQHHS SAHNKDIALG VFDVVVTDPS  980
CPASVLKCAE ALQLPVVSQE WVIQCLIVGE RIGFKQHPKY KHDYVSH                          1027
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**C**

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KPQTVAASSI YSMYTQQQAP GKNFQQAVQS ALTKTHTRGP HFSSVYGKPV IAAAQNQQQH PENIYSNSQG   70
KPGSPEPETE PVSSVQENHE NERIPRPLSP TKLLPFLSNP YRNQSDADLE ALRKKLSNAP RPLKKRSSIT  140
EPEGPNGPNI QKLLYQRTTI AAMETISVPS YPSKSASVTA SSESPVEIQN PYLHVEPEKE VVSLVPESLS  210
PEDVGNASTE NSDMPAPSPG LDYEPEGVPD NSPNLQNNPE EPNPEAPHVL DVYLEEYPPY PPPPYPSGEP  280
EGPGEDSVSM RPPEITGQVS LPPGKRTNLR KTGSERIAHG MRVKFNPLAL LLDSSLEGEF DLVQRIIYEV  350
DDPSLPNDEG ITALHNAVCA GHTEIVKFLV QFGVNVNAAD SDGWTPLHCA ASCNNVQVCK FLVESGAAVF  420
AMTYSDMQTA ADKCEEMEEG YTQCSQFLYG VQEKMGIMNK GVIYALWDYE PQNDDELPMK EGDCMTIIHR  490
EDEDEIEWWW ARLNDKEGYV PRNLLGLYPR IKPRQRSLA                                   529
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**D**

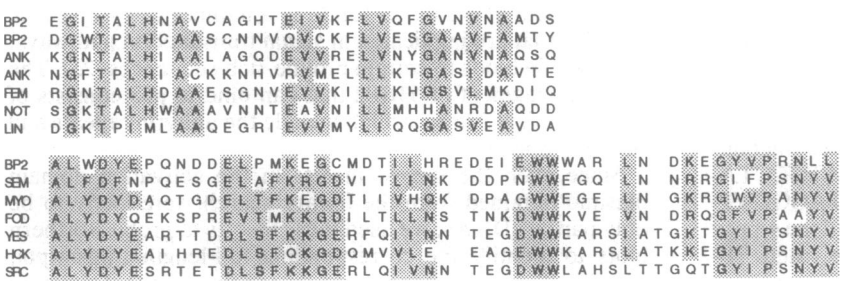


FIG. 1. Predicted amino acid sequences of two proteins that bind to p53. (*A*) Summary of the plasmids from the two-hybrid search that contained 53BP1 or 53BP2 inserts. Sizes of the cDNA inserts (in base pairs) up to the start of the poly(A) tail are shown. (*B*) Deduced protein sequence of the largest insert of 53BP1. (*C*) Deduced protein sequence of the largest insert of 53BP2. (*D*) Alignment of the ankyrin repeats (*Upper*) and Src homology 3 (SH3) domain (*Lower*) of 53BP2 with those of several other proteins. Identical and conserved residues found in at least five of the sequences are shaded. ANK, human ankyrin 1 (18); FEM, *Caenorhabditis elegans* sex-determining protein Fem-1 (19); NOT, *Drosophila* Notch protein (20); LIN, *C. elegans* Lin-12 protein; SEM, *C. elegans* Sem-5 protein (21); MYO, *Acanthamoeba* myosin heavy chain IB (21); FOD, human fodrin $\alpha$ chain (22); YES, chicken Yes protein-tyrosine kinase (23); HCK, mouse Hck protein-tyrosine kinase (23); SRC, human c-Src protein-tyrosine kinase (23).

the 53BP1 or 53BP2 hybrid to generate significant transcriptional activity; the two mutant p53 proteins paired with either 53BP1 or 53BP2 generated a transcriptional signal 100 times lower than the wild-type p53 (Table 1). However, the R273H mutant did show a very weak signal for interaction with 53BP2, a result consistent with evidence that this p53 mutant displays some features similar to those of the wild-type protein (26). As a control in the two-hybrid experiments, the mutant p53 proteins were capable of interacting with a wild-type murine p53 domain present as a DNA-binding-domain hybrid (Table 1).

***In Vitro* Binding of 53BP1 and 53BP2 to p53.** Since the signal in the two-hybrid assay could have been mediated by a yeast protein, we sought to confirm the interaction detected in the two-hybrid system with a biochemical assay. We used the C-terminal 270 aa of 53BP1 and 238 aa of 53BP2 fused to GST, because these fusion proteins were produced with less degradation than those with larger fragments. The GST fusions were purified from *E. coli* with glutathione-agarose beads. These beads, bound to either of the GST fusions or to GST itself, were mixed with a histidine-tagged full-length p53 generated from a baculovirus vector (17) or a histidine-tagged fragment of this p53 (aa 80–320) synthesized in *E. coli* and shown to be sufficient for site-specific DNA binding (17). These p53 proteins had been purified by metal affinity chromatography. The agarose beads complexed with GST or GST fusion and any bound p53 were pelleted and washed, and the bound and unbound protein fractions were analyzed by SDS/PAGE and Western blot analysis using antibody PAb240, which recognizes both mutant and wild-type p53 bound to nitrocellulose. GST did not bind full-length p53 or the p53 fragment (<3% of the total p53 recovered in the bound and unbound fractions) (Fig. 3). In contrast, GST–53BP1 bound 12% of the recovered full-length protein and 41% of the recovered p53 fragment, and GST–53BP2 bound 84% of the recovered full-length and 77% of the recovered p53 fragment (Fig. 3). This experiment indicates that 53BP1 and 53BP2 can bind directly to p53 and that the C-terminal oligomerization domain of p53, like the N-terminal transcriptional activation domain, is not necessary for the interaction with 53BP1 or 53BP2.

To test whether p53 could simultaneously bind to DNA in a site-specific manner and also bind to either 53BP1 or 53BP2, we assayed for the presence of DNA bound to p53 when the p53 was purified by virtue of its complexing with 53BP1 or 53BP2. Increasing amounts of the murine p53 fragment (aa 80–320) were mixed with a 28-bp $^{32}$P-labeled DNA containing a 20-bp consensus p53 binding site (27) in order to preform a

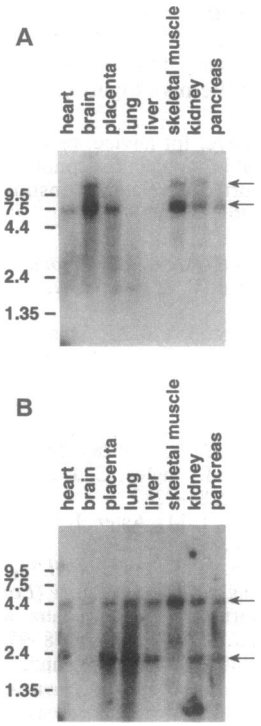


FIG. 2. RNA blot analysis of 53BP1 and 53BP2 expression in human tissues. A human tissue Northern blot was probed with a fragment of 53BP1 cDNA (*A*) and a fragment of 53BP2 cDNA (*B*). Each lane contained 2 μg of poly(A)$^+$ RNA from the tissues as indicated; size markers indicated on the left are in kilobases. Arrows indicate the positions of the hybridizing transcripts.

p53–DNA complex. The p53 was then isolated by adding either GST–53BP1 or GST–53BP2 bound to glutathione-agarose beads. As a positive control, Ni-NTA agarose beads, which directly bind the histidine-tagged p53 fragment, were added, and as a negative control GST alone bound to glutathione-agarose beads (which cannot isolate the p53) was added. The unbound protein and DNA were removed from the agarose-bound protein complexes by sequential washes, the proteins were eluted from the beads, and the DNA bound to p53 was quantified. Whereas the p53 fragment bound directly to Ni-NTA beads was complexed with up to 11,200 cpm of the input DNA, the p53 bound to either the 53BP1 or 53BP2 fusion was complexed with the same minimal amount of the site-specific DNA as the negative control (Fig. 4). We demonstrated by immunoblot that under the conditions of the DNA-binding assay, 53BP1 and 53BP2 bound comparable amounts of the p53 fragment as did the Ni-NTA agarose beads (Fig. 4). Although this experiment demonstrates that p53 cannot simultaneously bind the 53BP1 or 53BP2 GST-fusion and a DNA fragment with a specific binding site, it is possible that a full-length 53BP1 or 53BP2 protein might allow such simultaneous binding.

Table 1. Binding of wild-type and mutant p53 proteins to 53BP1 and 53BP2 in the two-hybrid assay

| | β-Galactosidase activity | | | |
|---|---|---|---|---|
| | | Activation-domain hybrid | | |
| DNA-binding-domain hybrid | Vector | Wild-type p53 | R175H p53 | R273H p53 |
| Vector | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.5 ± 0.3 | 0.2 ± 0.1 |
| 53BP1 | 0.2 ± 0.1 | 134 ± 37 | 0.3 ± 0.1 | 0.5 ± 0.3 |
| 53BP2 | 10.6 ± 2.0 | 3580 ± 105 | 8.8 ± 1.3 | 20.1 ± 2.2 |
| p53 | 0.2 ± 0.1 | 231 ± 60 | 39.7 ± 13 | 203 ± 63 |

Plasmids containing the indicated inserts used to transform a yeast reporter strain and β-galactosidase activity was determined with chlorophenol red β-D-galactopyranoside as substrate.

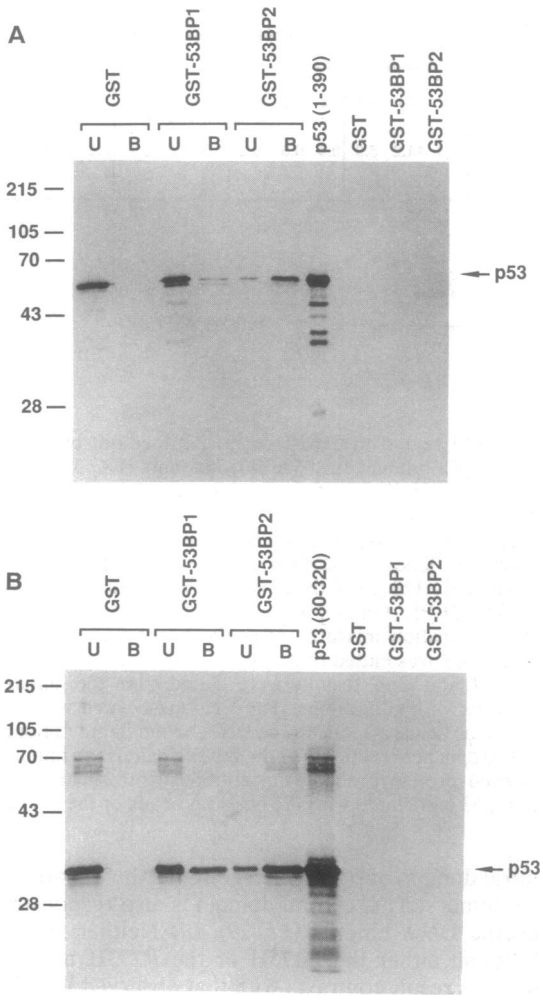


FIG. 3. Interaction of 53BP1 and 53BP2 with p53 *in vitro*. (*A*) GST, GST–53BP1, and GST–53BP2 were purified onto glutathione-agarose beads and incubated in ≈10-fold excess with 6 μg of full-length murine p53. The beads were pelleted and the supernatant was recovered as the unbound fraction (lanes U). The proteins were then eluted with glutathione and the eluates were pooled as the bound fraction (lanes B). The amount of p53 in the unbound and bound fractions was determined by immunoblot analysis using p53-specific antibody PAb240. The last four lanes contained aliquots of p53, GST, and the GST fusion proteins used in the binding experiment, to show the specificity of the antibody. (*B*) The same analysis was performed with a fragment of murine p53 (aa 80–320).

## DISCUSSION

We have identified two human proteins, 53BP1 and 53BP2, that bind to p53. These two proteins have not been identified by other assays—e.g., coimmunoprecipitation—and this may be due to the sensitivity of the two-hybrid system (28). Nevertheless, their likely relevance is suggested by the fact that the yeast assay did not yield a positive signal (apart from p53) with any of the other thousands of proteins tested in this search. Additionally, we assayed the GST–53BP1 and GST–53BP2 fusions for *in vitro* binding to six other control proteins and detected no binding (data not shown).

53BP1 and 53BP2 are notably different from other cellular proteins that bind to p53. (*i*) Unlike such proteins as MDM2, TBP, and RPA which bind to the N-terminal transcriptional activation domain of p53 (2–7) and therefore would not be detected in our two-hybrid search, 53BP1 and 53BP2 bind to

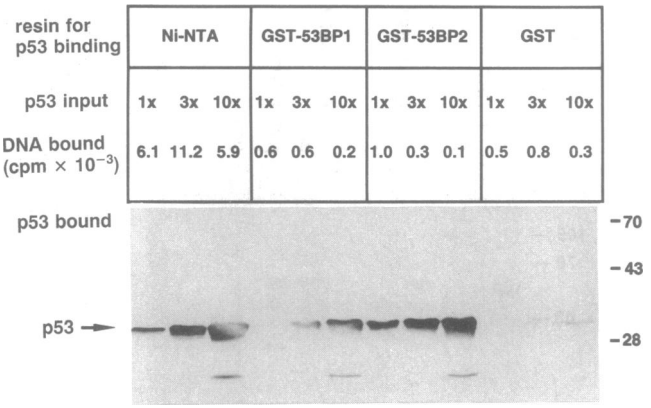


FIG. 4. p53 bound to 53BP1 or 53BP2 does not bind to DNA containing a p53 binding site. Various amounts (1×, 3×, and 10×, equal to 5, 15, and 50 μg) of the histidine-tagged p53 fragment (aa 80–320) were incubated with $^{32}$P-labeled DNA containing a p53-binding site. Ni-NTA agarose beads that directly bind to the histidine-tagged p53 (lanes Ni-NTA) or glutathione-agarose beads bound to approximately 120 μg of the 53BP1 or 53BP2 fusions or to GST (lanes GST–53BP1, GST–53BP2, and GST) were added to the p53/DNA incubation mixture. After the beads were pelleted and washed, the p53 was eluted from the Ni-NTA beads with imidazole and the GST and GST fusions were eluted from the glutathione-agarose beads with glutathione. The DNA associated with the p53 eluted from the beads is indicated as DNA bound (input radioactivity was 450,000 cpm per reaction). At the bottom panel is an immunoblot of the eluted protein probed with antibody PAb240 to show the amount of p53 associated with the Ni-NTA beads or the GST fusion proteins.

the central domain of p53, which contains the mutations that occur in tumors. This central domain is also responsible for site-specific DNA binding (17, 29). (*ii*) Neither 53BP1 nor 53BP2 bound either the R175H or the R273H mutant p53 protein. This result contrasts with that observed for MDM2, which binds to p53 that is mutant at residue 175 (2); RPA, which binds to both p53 mutants (6); and yeast TBP, which binds to p53 that is mutant at residue 273 but not to p53 that is mutant at residue 175 (5) [although Seto *et al.* (4) found that the yeast TBP did not bind to p53]. Our results indicate the likely requirement for p53 to be in its wild-type conformation for binding to these cellular proteins, analogous to what has been observed for p53 binding to simian virus 40 large tumor antigen (30, 31), and suggest that 53BP1 and/or 53BP2 is involved in some aspect of p53-mediated suppression. Large tumor antigen binds to the same domain of p53 as do 53BP1 and 53BP2, further suggesting that the viral protein could function in part by inhibiting the activity of either of these proteins.

That p53 does not appear to be capable of simultaneously binding to either 53BP1 or 53BP2 and to DNA carrying a consensus binding site suggests that these two proteins do not mediate transcriptional activation from this site through interactions with other components of the transcriptional machinery. However, 53BP1 or 53BP2 could be involved in determining the specificity of p53 binding to DNA sequences different from the consensus site. Alternatively, they might modulate the level of transcriptional activation, play a role in p53-dependent transcriptional repression, or regulate a p53 activity separate from transcription. The presence of ankyrin repeats and an SH3 domain in 53BP2 suggests that this protein might be involved in coupling p53 to cellular signaling events. While p53 has a number of potential functions in growth control, previously identified partners bind only to its transcriptional activation domain in a manner relatively insensitive to tumor-derived mutations. The identification of these two proteins that bind to the central domain of p53 opens avenues into the investigation of p53 activity.

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