A Novel Cellular Protein (MTBP) Binds to MDM2 and Induces a G$_1$ Arrest That Is Suppressed by MDM2*

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The MDM2 protein, through its interaction with p53, plays an important role in the regulation of the G$_1$ checkpoint of the cell cycle. In addition to binding to and inhibiting the transcriptional activation function of the p53 protein, MDM2 binds, inter alia, to RB and the E2F-1-DP-1 complex and in so doing may promote progression of cells into S phase. Mice transgenic for Mdm2 possess cells that have cell cycle regulation defects and develop an altered tumor profile independent of their p53 status. MDM2 also blocks the growth inhibitory effects of transforming growth factor-β1 in a p53-independent manner. We show here that a novel growth regulatory molecule is also the target of MDM2-mediated inhibition. Using a yeast two-hybrid screen, we have identified a gene that encodes a novel cellular protein (MTBP) that binds to MDM2. MTBP can induce G$_1$ arrest, which in turn can be blocked by MDM2. Our results suggest the existence of another growth control pathway that may be regulated, at least in part, by MDM2.

In tumors, loss of function either of p53 itself (1, 2) or of the p53-dependent pathway that activates G$_1$ arrest is one of the major and most frequent molecular events (reviewed in Ref. 3). p53 function may be compromised directly via mutation and/or deletion of the p53 gene (4) and indirectly by changes in the regulation or level of the MDM2 protein (5).

The Mdm2 gene, itself a transcriptional target of p53 (6–8), encodes a protein (MDM2) that is a critical negative regulator of p53 function (9, 10). Mdm2 was originally discovered as an oncogene that was amplified on mouse double minute chromosomes (11, 12). Mdm2 was later found to be amplified and overexpressed in a variety of human cancers (5, 13, 14). MDM2 binds to the transcriptional activation domain of p53 and thus inhibits this function of p53 (15, 16). Moreover, MDM2 binding to p53 regulates the stability of the p53 protein such that p53 is ubiquitinated and is then degraded by the proteasome (17, 18). This, together with the observed effect on p53 function, has led to a model in which an autoregulatory loop connects MDM2 and p53 (6, 8).

MDM2 inhibits both p53-mediated G$_1$ arrest and apoptosis (17, 19). p53 induces G$_1$ arrest by promoting transcriptional up-regulation of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ (20). Therefore, it is likely that MDM2 prevents p53 from inducing G$_1$ arrest by inhibiting p53-dependent transcriptional activation. MDM2 can prevent p53-mediated apoptosis, and this has been shown to be dependent on the ability of MDM2 to inhibit transcriptional repression by p53 (21). Moreover, a previously identified interaction with RB (22) was shown to be able to regulate this effect. By binding to MDM2, RB forms a stable ternary complex with p53, and this prevents the MDM2-promoted degradation of p53. The ternary complex can promote p53-dependent apoptosis, but not p53-mediated transactivation.

The autoregulatory relationship between p53 and MDM2 suggests that MDM2 overexpression may be oncogenic because of the resulting inactivation of p53 (8). This conclusion is supported by studies of human tumors that show that, in the majority of cases, either p53 is mutated/deleted or MDM2 is overexpressed (23). That a primary function of Mdm2 is indeed its ability to negatively regulate p53 is further supported by studies of allelic knockouts of these genes in mice. Mice that possess a homozygous deletion of Mdm2 die at around day 5 of embryogenesis, whereas mice that possess a homozygous deletion of both Mdm2 and p53 are viable and develop normally (24, 25). No differences have been detected between these p53$^{-/-}$ and p53$^{-/-}$/Mdm2$^{-/-}$ mice in terms of the rate or spectrum of tumors developed (26). Also, no differences could be detected between the embryonic fibroblasts derived from these animals in terms of their growth or cell cycle characteristics.

Collectively, these observations might suggest that the only function of Mdm2 is to regulate p53 activity, and perhaps during normal development, this is indeed the case. However, the situation appears to be different when MDM2 is expressed at abnormally high levels. Experiments in which MDM2 was overexpressed in NIH3T3 cells have shown that naturally occurring splice variants of MDM2 that lack the ability to bind to p53 are still able to transform these cells (27). Further support for the idea that MDM2 has p53-independent effects derives from studies of transgenic mice. Mice transgenic for an Mdm2 gene expressed from a β-lactoglobulin promoter exhibited abnormal mammary development, with cells becoming polypliod together with a multinucleate morphology, suggestive of DNA synthesis in the absence of mitosis (28). The same results were obtained in both wild-type p53 animals and animals with a homozygous deletion of p53. In addition, recent studies using a different transgenic system with multiple copies of the whole Mdm2 gene being used to generate mice that overexpress...
MDM2 from the Mdm2 promoter have shown that these animals develop a different spectrum of tumors, cf. p53 null mice (29). The same effect of MDM2 overexpression was observed regardless of the p53 status of these animals. Finally, in support of the existence of p53-independent effects of MDM2 upon overexpression, it has recently been shown that Mdm2 has the ability to abrogate the growth inhibitory activities of transforming growth factor-β1. This effect was p53-independent in cells in culture (30). Taken together, these results all suggest that overexpression of MDM2 acts not only upon p53, but also on additional pathways. Since the mechanism(s) by which MDM2 exerts these p53-independent effects have not yet been elucidated, we have tried to identify novel MDM2-binding proteins that could help us to understand how MDM2 overexpression alters cell growth regulation. Using MDM2 as the bait in a yeast two-hybrid screen, we identified several novel MDM2-binding proteins. Further screening of these enabled us to focus on one novel gene that encodes a protein that we have called MTBP for MDM2 (two)-binding protein. Our results show that MTBP is capable of negatively regulating growth by inducing G1 arrest in a p53-independent manner and, moreover, that this can be suppressed by MDM2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Antibodies**—Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin/neomycin (Life Technologies, Inc., H1299 (ATCC CRL-5803), U2OS (ATCC HTB-96), and Saos-2 (ATCC HTB-85) cells were obtained from American Type Culture Collection. pGal4-DDB-MDM2 encodes full-length mouse MDM2 cloned in-frame with the Gal4 DNA-binding domain (DBD)

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and gat atc tac gat tca tag atc tc-3
and gat gag cta cct gg-3

from pCEP-MTBP (see below) into the NotI site of pBBV. The pSK-MTBP construct used for screening of these enabled us to focus on one novel gene that encodes a protein that we have called MTBP for MDM2 (two)-binding protein. Our results show that MTBP is capable of negatively regulating growth by inducing G1 arrest in a p53-independent manner and, moreover, that this can be suppressed by MDM2.

**In Vitro Binding and Immunoassays**—For in vitro binding assays, MDM2 and Δ166-MDM2 were expressed in XL-1 bacteria (Stratagene) from the pQE32-DM2 and pQE31-Δ166-MDM2 constructs, respectively; captured on Ni²⁺-agarose (QIAGEN Inc.); and washed with 40 mM imidazole. Beads were then resuspended in loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography using the Aerolift Method (Amersham Pharmacia Biotech).

Cells were transfected either by the calcium phosphate-DNA coprecipitation method (34) or using FUGENE-6™ (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For immunoprecipitation experiments, cells were typically transfected with 10 μg of each plasmid, and proteins were extracted 48–72 h post-transfection. Transfected cells were harvested, and the cell pellet was lysed in immunoprecipitation buffer: lyse buffer (50 mM Tris, pH 7.4, 10% glycerol, 0.1% Triton X-100, and 0.5 mg/ml bovine serum albumin) in the presence of protease inhibitors (1–2 μg/ml aprotinin, 1–2 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml soybean trypsin inhibitor (Roche Molecular Biochemicals), and 1 mM phenylmethylsulfonyl fluoride) for 10 min on ice. The lysate was clarified by centrifugation for 10 min at 4 °C, and the concentration of total proteins was determined by the Bio-Rad protein assay. Between 1 and 5 mg of protein were then precleared by incubation with 50 μl of protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. The precleared lysate was incubated with 1 μg of primary antibody for 1 h at 4 °C, followed by incubation with 50 μl of protein G-Sepharose for 2 h at 4 °C. Immunoprecipitated complexes were washed three times with immunoprecipitation buffer; resuspended in 30 μl of protein sample buffer (0.1% Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 0.5% dithiothreitol); and subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to Hybond ECL membrane (Amersham Pharmacia Biotech). Following incubation with primary antibodies and subsequently with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech), the signals were detected by enhanced chemiluminescence with Renaissance™ (NEN Life Science Products).

**FACS, Cell Cycle Analysis, and Colony Assays**—Saos-2 and U2OS cells were transfected using FUGENE-6 with the indicated plasmids. Cells were harvested and analyzed by FACS essentially as described (19). Briefly, nucodazole was added to the indicated cells at 50 ng/ml 12 h prior to harvesting. Cells were harvested 48–72 h after the addition of FUGENE-6 DNA complexes and washed with Dulbecco’s phos-

2 The abbreviations used are: DBD, DNA-binding domain; AD, activation domain; HA, hemagglutinin; RACE, rapid amplification of cDNA ends; FACS, fluorescence-activated cell sorting.
cDNA from a novel gene fused to the activation domain of Gal4 (Gal4-AD-3’-MTBP) interacted with Gal4-DBD-MDM2, but not with Gal4-DBD. To confirm this interaction in a different system, the in vitro translated cDNA from the yeast two-hybrid screen (pBBV-3’-MTBP) was mixed with recombinant His-tagged MDM2. Fig. 1B shows that pBBV-3’-MTBP encoded a peptide that can bind in vitro to MDM2. Sequence analysis of this cDNA demonstrated that it is a novel sequence that encodes a predicted peptide of 380 amino acids. Northern analysis demonstrated that the carboxyl-terminal cDNA hybridized to a 3 kilobases (Fig. 2A) and data not shown). We therefore cloned the rest of the cDNA for this gene using a RACE-based strategy. Analysis of 5’-RACE products from mRNA obtained from a murine B-cell line demonstrated that several clones possessed an authentic 5’-end; they were identical and terminated upstream of a single long open reading frame that was in frame with the clone identified in the yeast two-hybrid screen. The sequence of this clone has been deposited in the GenBankTM/EBI Data Bank (AJ278508). This cDNA encodes a protein with a predicted molecular mass of 104 kDa, and we have given this gene the name Mtbp (MDM2 (two)-
binding protein). Data base analysis detected two yeast genes whose protein products possessed significant homology to MTBP: BOI1 and BOI2 (36, 37). The two proteins encoded by these genes (Boi1p and Boi2p, respectively) exhibit an overall amino acid identity of 38%, but this is concentrated into four regions (I–IV) that possess identities of 71, 65, 78, and 69%, respectively. Both Boi1p and Boi2p inhibit growth in yeast when expressed at high levels. The homology between Boi1p and MTBP and between Boi2p and MTBP is 21.2 and 21% amino acid identities in alignments of 401 and 400 amino acids, respectively, and is entirely contained within the carboxy-terminal regions of all three proteins. Fig. 1C shows the FASTA-generated alignment of Boi2p and MTBP. Domain 3 of Boi2p is a proline-rich area that is essential for binding to the second SH3 region of Bem1p. The corresponding region of MTBP is also proline-rich. It is noteworthy that the growth inhibitory function of Boi2p is entirely contained within the carboxy-terminal moiety of the protein. Apart from expressed sequence tags, no other substantial homologies to MTBP could be identified.

The full-length cDNA for MTBP was then used to examine the pattern of expression of this gene by Northern blotting. Fig. 2A shows that MTBP was expressed in a variety of normal tissues, with the highest levels of expression in the thymus, testis, and ovary and with low or almost undetectable expression in peripheral blood lymphocytes. We also found MTBP expression in the pancreas, heart, liver, skeletal muscle, and lung and relatively low expression in the brain (data not shown).

To test whether the interaction we had detected between the carboxy-terminal region of MTBP and MDM2 also occurred with the full-length form of the protein, we performed an in vitro binding assay using recombinant His6-MDM2 and in vitro translated MTBP as shown in Fig. 2B. Further confirmation of the interaction between these two proteins came from our analysis of mammalian cells transfected with MDM2 and a carboxy-terminal HA-tagged form of MTBP. Immunoprecipitation with either an anti-HA or anti-MDM2 monoclonal antibody, followed by Western blot analysis, demonstrated that the two proteins could be coprecipitated as shown in Fig. 2C. These results suggest that a novel protein (MTBP) can bind specifically to MDM2 under these conditions.

Identification of the Region of MDM2 Required for Binding to MTBP—The MDM2 protein has a number of highly conserved regions, and the function of these is not fully understood (reviewed in Ref. 38). To determine the region of MDM2 that binds to MTBP, we used a series of carboxy-terminal deletion mutants of MDM2 to identify the region required for binding to MTBP. Yeast were transformed with a GAL4-DBD-MDM2 construct possessing either full-length MDM2 (amino acids 1–489) or a series of deletion mutants as indicated, together with either a GAL4-AD or GAL4-AD-3′-MTBP construct essentially as described for Fig. 1A. Transformed yeast cells were grown overnight in Leu/Trp-free medium and then inoculated onto both Leu/Trp-free medium (not shown) and Leu/Trp/His-free medium (shown) to confirm transformation with both constructs and interaction, respectively. B, an in vitro binding assay was performed essentially as described for Fig. 1B. In addition to testing for binding to MDM2 and XL-1 cells, we tested for binding to a deletion mutant of MDM2: Δ166. Left panel, binding of the in vitro translated carboxy-terminal 380 amino acids of MDM2 (pBBV-3′-MTBP); right panel, binding of full-length in vitro translated MTBP. Both full-length MTBP and the carboxy-terminal mutant bound to MDM2 and Δ166, but not to the XL-1 negative control. IVT indicates 10% of the amount of in vitro translated input for protein binding. C, shown is a diagrammatic representation of the MDM2 protein, with several motifs and the p53-binding region indicated. The area of MDM2 that is sufficient for binding to MTBP (amino acids 167–304) is also indicated. NLS, nuclear localization signal; NES, nuclear export signal; Acidic, a highly acidic region; Zn, a zinc finger; Zn/Ring, a zinc ring finger.

Fig. 3C, this portion of MDM2 contains a nuclear localization signal, a motif identified as a nuclear export signal, and an acidic region. Our results in both yeast (Fig. 3A) and in vitro (Fig. 3B, left panel) suggest that the carboxy-terminal portion of MDM2 (amino acids 515–894) is sufficient for binding to MTBP. We conclude that MDM2 binds to the carboxy-terminal 380 amino acids of MTBP and that an area of MDM2 bounded by amino acids 167–304 is sufficient for the binding interaction to occur.

MTBP Inhibits Cell Growth—Several MDM2-binding proteins are regulators of cell growth; and indeed, both of the MTPB partial homologs (Boi1p and Boi2p) have been shown to have growth inhibitory activity (36, 37). Therefore, we investigated the effect of MTBP expression on cell growth in culture. Fig. 4A shows that when an expression construct for MTBP was transfected into U2OS cells, no colonies were produced, in
MDM2 Suppresses MTBP-induced G1 Arrest

**MTBP Induces a p53-independent G1 Arrest**—We performed cell cycle analysis to examine the possibility that MTBP might act at a specific point in the cell cycle. Cells were cotransfected with an expression construct for CD20 so that only the transfected cell population need be analyzed. At any given time in a rapidly cycling population of the cells we have used, typically 50% will have a 2N DNA complement. Our initial experiments suggested that MTBP expression induces an increase in the percentage of cells with a 2N DNA complement (data not shown), and we therefore set out to examine this further. To facilitate detection of effects in this stage of the cell cycle, we treated cells with the microtubule-disrupting drug nocodazole. In Fig. 5A, the result of MTBP expression in U2OS cells can be compared with cells that were transfected by the vector alone and also with cells transfected with p53 from the same vector. As expected, p53 expression induced an increase in the percentage of cells in G1 from 15.9 to 24.9%. MTBP expression induced a similar effect with an increase to 24.0%. As with the growth inhibition that we had detected, the possibility existed that the effect we observed of MTBP expression on the cell cycle was in some manner dependent on p53. To examine this, we performed a similar experiment with H1299 cells. Fig. 5B shows that both p53 and MTBP induced a comparable increase in the percentage of cells with a 2N DNA content from 22.4 to 35.8 and 38.3%, respectively. These experiments were performed on at least three occasions, and similar results were obtained each time. From these results, we conclude that p53 is not required for MTBP-mediated cell cycle arrest. We also examined the effect of MTBP expression on Saos-2 cells since we had found them to be resistant to the growth inhibitory effect of MTBP expression. Analysis of these cells clearly demonstrated that expression of MTBP had no effect on their cell cycle, and this is shown in Fig. 5C. These results suggest that the ability of MTBP to inhibit colony formation is consistent with its ability to alter the cell cycle. From these experiments, we conclude that MTBP induces G1 arrest in a p53-independent manner.

**MDM2 Suppresses the G1 Arrest Induced by MTBP**—Since MDM2 blocks p53-mediated cell cycle arrest (19), we investigated whether it might not also be able to inhibit the effect of MTBP. As shown in Fig. 6A, MDM2 expression resulted in complete abrogation of the effect of MTBP in U2OS cells. Fig. 4 shows that MTBP had no effect, whereas p53 reduced the level of β-galactosidase expression. We also performed a similar analysis in a more quantitative fashion using FACS and observed no reduction in the number of positive cells or signal strength of CD20 when cotransfected with MTBP. We again saw a 10% reduction in both with p53 (data not shown).

An alternative explanation for the growth inhibitory effect we observed in U2OS cells could be the activation of p53. In these cells, p53 is wild type, but is transcriptionally inactive because of the presence of high levels of MDM2 (39). Thus, transfection of MTBP might simply compete with p53 for binding to MDM2 and in so doing release the MDM2-mediated block. To examine this possibility, we measured the levels of p53 itself and of the p53 transcriptional target p21Waf1/Cip1 (40) in the presence of MTBP. As shown in Fig. 4D, we saw no alteration in the levels of either of these proteins. Taken together, these observations suggest that high level expression of MTBP has a negative effect on cell growth and that this is independent of p53.

**MTBP Suppresses the G1 Arrest Induced by MDM2**—MDM2 expression resulted in complete abrogation of the effect of MTBP in U2OS cells. The availability of other transcription factors. To investigate this, we cotransfected H1299 cells with a β-galactosidase expression construct and measured the levels of β-galactosidase by Western blotting in the presence of either the MTBP or p53 expression constructs and also with the pCEP vector. Fig. 4C shows that MTBP had no effect, whereas p53 reduced the level of β-galactosidase expression. We also performed a similar analysis in a more quantitative fashion using FACS and observed no reduction in the number of positive cells or signal strength of CD20 when cotransfected with MTBP. We again saw a 10% reduction in both with p53 (data not shown).

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shows that at this ratio of MDM2 to MTBP, there was little
effect on the level of MTBP protein. Thus, we conclude that
MDM2-mediated inhibition of the MTBP-induced cell cycle ar-
rest does not require degradation of MTBP. We cannot infer
from these results that direct binding of MTBP by MDM2 is
required for inhibition of MTBP because we cannot rule out the
possibility that MDM2 simply acts downstream of MTBP. We
conclude that MDM2 suppresses the G1 arrest mediated by
MTBP; and since this does not require degradation of MTBP, it
seems likely that the effect is a consequence of the ability of
MDM2 to bind directly to MTBP.

**DISCUSSION**

We sought to investigate the function of the MDM2 protein
by identifying novel MDM2-binding proteins. In particular, we
wanted to identify p53-independent pathways by which MDM2
may exert some of its effects. We identified a number of candi-
date MDM2-binding proteins, one of which is a novel protein
that we have named MTBP. MTBP has some homology to two
yeast proteins (Boi1p and Boi2p) that are involved in cell cycle-
regulated events in *S. cerevisiae* and that can bind to an SH3
domain. We do not know whether the homologous region of
MTBP binds to SH3 domains, but given that many SH3-bind-
ing proteins use a proline-rich section of amino acid residues
for binding (41), this would appear to be a reasonable possibil-
ity. No other significant homology to MTBP could be identified
on the basis of data base analyses of the primary sequence.
Numerous sequence motifs were identified within MTBP, as
might be expected for a peptide of 894 amino acids. In partic-
ular, six potential nuclear localization signals were detected
(both mono- and bipartite), which would be compatible with a
nuclear localization for this protein. Northern analysis showed
that MTBP expression can be detected in a wide variety of
tissues, with the highest levels of expression in the thymus,
testis, and ovary. These three tissues are sites of high levels of
cell proliferation and differentiation and, moreover, are the
same tissues that exhibit the highest levels of expression of
MDM2 (12). However, the similarity in the pattern of expres-
sion of these two genes is not complete; for example, MDM2 is
expressed at high levels in the brain, and MTBP is not.

We initially identified a cDNA that corresponds to amino
acids 515–894 of MTBP and confirmed that both this fragment
and the full-length protein bound to MDM2 in an *in vitro*
assay. This suggests that this interaction is likely to be direct. Further
confirmation that these two proteins interact was obtained
from immunoprecipitation experiments. The results from these

![Fig. 5. MTBP induces G1 arrest in a p53-independent manner.](image)

**A** U2OS cells were transfected with 5 μg of the indicated plasmids together with 2 μg of pCMVNeoBam-CD20 using FUGENE-6 and were harvested 60 h after the addition of DNA. 12 h before harvesting, cells were treated with nocodazole at a final concentration of 50 ng/ml. Cells were stained first with an anti-CD20 antibody (leu16) and then with fluorescein isothiocyanate-conjugated anti-mouse IgG and were finally stained with propidium iodide before analysis. Typically 20% of the cells stained positive for CD20, and these were then analyzed. **B** H1299 cells were transfected with 5 μg of the indicated plasmids and selected in hygromycin B for 3 days. Cells were washed and refed with antibiotic-free medium and treated with nocodazole at a concentration of 20 ng/ml for 16 h prior to harvesting. Cells were fixed and stained with propidium iodide. **C** Saos-2 cells were transfected with 5 μg of the indicated plasmids together with 2 μg of CD20 expression construct and analyzed as described for A, except that cells were treated with nocodazole at a concentration of 50 ng/ml for 16 h prior to harvesting.

![Fig. 6. MDM2 suppresses the G1 arrest induced by MTBP.](image)

**A** U2OS cells were transfected as indicated with 1 μg of pCEP, p53, or MTBP and cotransfected with 10 μg of pCMV or pMDM2 as indicated together with 2 μg of pCMVNeoBam-CD20. Cells were transfected using FUGENE-6 and harvested 60 h after the addition of DNA. 12 h before harvesting, cells were treated with nocodazole at a final concentration of 50 ng/ml. Cells were stained first with an anti-CD20 antibody and then with fluorescein isothiocyanate-conjugated anti-mouse IgG and were finally fixed and stained with propidium iodide before analysis. Typically 20% of the cells stained positive for CD20, and these were then analyzed. **B** U2OS cells were transfected as indicated with 1 μg of MTBP and 10 μg of either pCMV or pMDM2 (pCMVNeoBam-MDM2). Cells were harvested 60 h after the addition of DNA and analyzed by Western blotting. MTBP was detected using anti-HA antibody 16B12.
demonstrated that both proteins can be coprecipitated from cell lysates using either anti-MDM2 or anti-HA (MTBP) monoclonal antibodies for the immunoprecipitation. Using this system, however, we cannot distinguish between a direct and an indirect interaction. Using both yeast and in vitro analyses, we have determined that the interaction of the carboxyl-terminal 380 amino acids of MTBP with MDM2 requires an area of MDM2 that lies between amino acids 167 and 304. This is a busy region of MDM2 since it overlaps with the MDM2 binding sites for p300, transcription factor IIE, RB, and p19Arf (21, 22, 42–45). The p300-binding region of MDM2 lies between amino acids 102 and 222, and since p300 binding to MDM2 has been shown to be necessary for MDM2-mediated degradation of p53, the possibility exists that MTBP binding to MDM2 might block this (42). To date, we have not seen such a blocking effect of MTBP on MDM2-mediated degradation of p53. The region responsible for interaction of MDM2 with the 34-kDa subunit of transcription factor IIE lies between MDM2 amino acids 50 and 222 (45). This interaction has been implicated in the ability of MDM2 to function as a transcriptional repressor. We do not yet know whether MTBP affects MDM2 binding to transcription factor IIE or the ability of MDM2 to act as repressor of transcription. The portion of MDM2 responsible for binding to RB (amino acids 272–320) also overlaps with the MTBP-binding region (21, 22). It has recently been shown that by binding to MDM2, RB (preferentially the hypophosphorylated form) can form a ternary complex with p53 that is distinct from the p19Arf–MDM2–p53 complex and appears to perform a distinct function (21). Experiments to determine whether MTBP can compete with RB or transcription factor IIE for binding to MDM2 are in progress. p19Arf binds to an area of MDM2 that lies between amino acids 154 and 221 (plus a further interaction point contained with the carboxyl-terminal 271 amino acids) (43, 44) and in so doing prevents MDM2 from targeting p53 for degradation. As stated above, we have not yet seen any effect of MTBP on MDM2-mediated degradation of p53; and thus, it appears that MTBP may not be able to compete with p19Arf for binding to MDM2. In addition, the MTBP-binding region of MDM2 contains one of two nuclear localization signals and a leucine-rich nuclear export signal (reviewed in Ref. 38). Clearly, we will need to perform more detailed studies to determine more accurately the precise binding site on MDM2 and also the effects of that binding on the localization and function of each of these proteins.

While trying to establish stable cell lines that express high levels of MTBP, we observed that MTBP expression resulted in a significant reduction in the number of colonies we obtained. This effect was not entirely unexpected because of the apparent homology between MTBP and the yeast genes BOI1 and BOI2. We show here that this suppression of cell growth is the result of an arrest in the G1 phase of the cell cycle induced by expression of MTBP. We do not know the mechanism by which MTBP exerts its cell cycle inhibitory activity. Our experiments in H1299 cells show that the effect of MTBP is not dependent on p53; and moreover, our experiments in Saos-2 cells suggest that MTBP may perhaps act upstream of RB. Experiments to investigate this are under way. We have not detected a dramatic increase in the population of cells that possess a sub-2N DNA complement, typically indicative of apoptosis, when MTBP is expressed at high levels. We note, however, that in these cells, we also did not see a dramatic increase in the sub-2N DNA complement of cells transfected with p53. Nevertheless, the growth inhibition that we have seen appears to be entirely the result of cell cycle arrest and not due to a combination of arrest and active cell death. We have noted, however, in Saos-2 cells, which lack both p53 and RB, that although there is no effect on the cell cycle profile, there is an ~3–4-fold reduction in colony formation. This may suggest that expression of MTBP has additional, perhaps more subtle effects on cell growth.

MDM2 expression clearly inhibits the ability of MTBP to induce cell cycle arrest. Our data suggest that MDM2 does not induce degradation of MTBP, and this observation supports the notion that binding of MDM2 to MTBP may be sufficient for MDM2-mediated inhibition of MTBP-induced cell cycle arrest. Formal proof that MDM2 binding to MTBP is required for inhibition will necessitate the identification of mutants of MDM2, within the MTBP-binding region, that do not bind to MTBP and consequently do not inhibit MTBP-mediated growth arrest.

Our data led us to suggest that the normal function of MTBP may be to regulate cell growth; and therefore, we need to determine the signals that regulate the activity of MTBP. One piece of information that may shed light upon this question is the homology between MTBP and Boi proteins. In yeast, these proteins are part of a pathway that is required for maintenance of cell polarity, which is necessary for bud formation. This pathway is regulated by Cdc42p, a member of the Rho family of GTPases, together with an associated GTP-GDP exchange factor, Cdc24p (reviewed in Ref. 46). We therefore speculate that MTBP may play a role in the regulation of a Cdc42p-dependent pathway.

An alternative possibility is suggested by the interaction of MTBP with MDM2. The primary function of MDM2, under most circumstances, appears to be to promote cell cycle progression (19, 22, 47). This is supported by studies of the effects of the interaction of MDM2 with p53 and with E2F-1–DP-1, where MDM2 has either an inhibitory or stimulatory effect, respectively, which is consistent with the function of the individual protein target. MDM2 interacts with a number of tumor suppressor proteins (p53, E2F-1, RB, and p19Arf), which when expressed at high levels can induce growth arrest in vitro (48–50), similar to that induced by the MTBP protein. The possibility therefore exists that MTBP may be a tumor suppressor protein.

Numerous cellular insults and stimuli induce p53 activity. Since there is no evidence for the existence of a functional DNA damage-inducible G1 checkpoint in p53 null cells (reviewed in Ref. 51), we can rule out the possibility that MTBP plays any growth inhibitory role in such a pathway, unless MTBP function in vivo also requires p53 function. This seems unlikely because MTBP is not a transcriptional target of p53. Therefore, it is more likely that MTBP plays a role in response to some other signal.

When expressed at high levels, MDM2 plays a role in p53-independent pathways that impact upon cell cycle regulation and tumorigenesis. It remains to be seen whether the role of MDM2 in these pathways in a physiological setting is dependent on p53-mediated up-regulation of MDM2. Certainly this is unlikely to be the case when MDM2 expression is increased following amplification of the Mdm2 gene. MDM2 can abrogate the cell growth inhibitory effects of transforming growth factor–β1 in a p53-independent manner, and this effect feeds into the RB-dependent G1 checkpoint pathway. Whether the interaction between MDM2 and MTBP plays a part in these events is unclear.

The results presented here show that a novel cellular protein (MTBP) binds to MDM2 and induces G1 arrest when expressed

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2 D. S. Haines, unpublished data.

3 M. T. Boyd, unpublished data.
at high levels and that this arrest can in turn be inhibited by MDM2. MTBP may thus provide an additional link between MDM2 function and regulation of the G1 cell cycle checkpoint.

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REFERENCES

1. Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., and Harris, C. (1996) *Nature* **381**, 318–324
2. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, M., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., and Heddle, N. (1993) *Nature* **362**, 573–577
3. Dasmahapatra, B., Rozhon, E. J., and Schwartz, J. (1987) *Science* **236**, 705–708
4. Fields, S., and Song, O. (1989) *Science* **249**, 447–453
5. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, Cold Spring Harbor, NY
6. Fields, S., and Song, O. (1989) *Nature* **340**, 245–246
7. Bender, A. R., Chen, J., Meltzer, P. S., George, D. L., and Vogelstein, B. (1996) *J. Cell Biol.* **133**, 865–878
8. Borer, J., Wu, L., and Levine, A. J. (1992) *Science* **255**, 1237–1245
9. Attardi, L. D., and Jacks, T. (1999) *Science* **285**, 1237–1245
10. Cohn, M., Liu, S., Chang, W., and Englund, E. (1992) *Science* **256**, 705–708
11. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995) *Nature* **378**, 206–208
12. Haupt, Y., Barak, Y., and Oren, M. (1996) *Nature* **381**, 713–717
13. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
14. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *EMBO J.* **12**, 3011–3016
15. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
16. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
17. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
18. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
19. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
20. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
21. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
22. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
23. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
24. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
25. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
26. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
27. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
28. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132
29. Haupt, Y., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993) *Genes Dev.* **7**, 1126–1132