Repression of p53-mediated transcription by MDM2: a dual mechanism

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The oncoprotein MDM2 binds to the activation domain of the tumor suppressor p53 and inhibits its ability to stimulate transcription. This same region of p53 is able to bind several basal transcription factors that appear to be important for the transactivation function of p53. It has therefore been suggested that MDM2 acts to inhibit p53 by concealing its activation domain from the basal machinery. Here we present data suggesting that MDM2 possesses an additional inhibitory function. Our experiments reveal that in addition to a p53-binding domain, MDM2 also contains an inhibitory domain that can directly repress basal transcription in the absence of p53. By fusing portions of MDM2 to a heterologous DNA-binding domain to allow p53-independent promoter recruitment, we have localized this inhibitory domain to a region encompassing amino acids 50–222 of MDM2. Furthermore, the function of this inhibitory domain does not require the presence of either TFIIB or the TAFs. Of the remaining basal factors, both the small subunit of TFIIE and monomeric TBP are bound by the MDM2 inhibitory domain. It is possible that MDM2 inhibits the ability of the preinitiation complex to synthesize RNA through one of these interactions. Our results are consistent with a model in which MDM2 represses p53-dependent transcription by a dual mechanism: a masking of the activation domain of p53 through a protein–protein interaction that additionally serves to recruit MDM2 to the promoter where it directly interferes with the basal transcription machinery.

[Key Words: MDM2; p53; in vitro; transcriptional inhibitor; basal transcription]

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One of the primary biological functions of p53 is to prevent the accumulation of genomic alterations after DNA damage. To achieve this goal, p53 inhibits the growth of injured cells by two mechanisms (Haffner and Oren 1995; Ko and Prives 1996). In response to damaged DNA, p53 is capable of mediating a G1 cell cycle arrest, presumably to allow the cell time to make necessary genomic repairs. p53 can also cause the elimination of damaged cells by initiating the process of programmed cell death. The ability of p53 to induce a G1 arrest is dependent on its ability to function as a site-specific transcriptional activator (Fields and Jang 1990; Raycroft et al. 1990; Farmer et al. 1992; Kern et al. 1992; Unger et al. 1992; Zambetti et al. 1992; Attardi et al. 1996) and to induce the expression of genes whose products are themselves directly involved in arresting cell growth and proliferation (for review, see Ko and Prives 1996). This cell cycle arrest is mediated by increasing expression of the p21 protein, a cyclin-dependent kinase inhibitor (el-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Dulic et al. 1994; Brugarolas et al. 1995; Deng et al. 1995). In addition, the product of another p53-response gene, GADD45, may play some role in the p53-dependent G1 arrest (Smith et al. 1994). The role of the transactivating function of p53 in the induction of apoptosis is less clear. However, the p53-response gene bax has been suggested to be involved in this process (Miyashita and Reed 1995). Taken together, these findings demonstrate the importance of the transcriptional activation properties of p53 for its ability to suppress tumor development and growth.

Because of its critical role in the control of cell growth and proliferation, the expression of p53 itself must be tightly regulated. Numerous modulators of the function of p53 have been reported, including kinases (Hupp et al. 1992; Takenaka et al. 1995), components of the ubiquitin-degradative pathway (Schneffner et al. 1993; Chowdary et al. 1994), virally encoded proteins (Bargonetti et al. 1992; Schneffner et al. 1993; Wang et al. 1994), and transcriptional inhibitors (Momand et al. 1992; Oliner et al. 1992, 1993; Yew et al. 1994). One crucial cellular regulator of p53 is the oncoprotein MDM2. Its ability to regulate p53 activity was first suggested when the mdm2 gene was found to be amplified in human sarcomas (Oliner et al. 1992) and the encoded protein was found to interact directly with p53 (Momand et al. 1992; Oliner et al. 1992). In transfection experiments, MDM2 can repress both p53-dependent transactivation of a reporter gene (Momand et al. 1992) and p53-depen-
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Effect on p53 regulation. In these experiments, mdm2 null mice were found to possess an embryonic lethal phenotype that was completely relieved when these mdm2−/− mice were produced in a p53 null background (de Oca Luna et al. 1995; Jones et al. 1995). These results suggest that the primary defect in mdm2−/− mice is an excess of p53 activity. For these reasons, an understanding of how p53 functions to regulate growth in vivo will require that we also understand how p53 is regulated by MDM2.

Although it is clear that MDM2 is an important modulator of p53 function, the mechanism by which MDM2 inhibits p53 is not well understood. The current model for the inhibitory effects of MDM2 on p53-dependent transcription suggests that MDM2 disrupts the ability of p53 to make important contacts with the general transcription machinery through direct binding and concealment of its activation domain (Oliner et al. 1993). In support of this hypothesis, a truncated MDM2 protein, lacking the first 49 amino acids, that is no longer capable of binding p53 is not capable of inhibiting p53-dependent activation in transfection experiments (Haines et al. 1994). Furthermore, several components of the transcription machinery have been found to bind to the activation domain of p53 in a region near the MDM2 binding site (Seto et al. 1992; Chen et al. 1993; Liu et al. 1993; Truant et al. 1993; Xiao et al. 1994; Lu and Levine 1995). Together, these results suggest that the critical interaction between MDM2 and the activation domain of p53 may disrupt important interactions between p53 and the basal machinery.

Although the masking theory of p53 inhibition by MDM2 is consistent with the mutant and in vitro binding data, this mechanism has not been formally demonstrated. In addition, other transcriptional repressors that bind to transactivators are known and have been suggested to inhibit transcription via other mechanisms. For instance, both the adenovirus E1B and the cellular retinoblastoma (Rb) protein appear to contain inhibitory domains that are targeted to the appropriate promoters by interacting with p53 and E2F, respectively (Yew et al. 1994; Weintub et al. 1995). Once at the promoter, they may inhibit additional transactivators or may directly repress the activities of the basal transcription machinery. It is possible that MDM2 may also function in a similar manner and that the interaction with p53 serves to recruit an inhibitory domain to p53-responsive promoters. In this case, MDM2 would be expected to contain a separable repression domain capable of inhibiting transcription when brought to a promoter independently of p53. This model for MDM2 inhibition is not mutually exclusive with the current, concealment model for repression by MDM2. As will be discussed later, MDM2 may repress transcription through a dual mechanism, by directly blocking p53 activation and by inhibiting activation by other transactivators bound at p53-responsive promoters.

At the present time, the specific mechanisms by which transcriptional repressors function are not well understood. Previous studies of transcriptional regulation in mammalian systems have focused primarily on transcriptional activators, and relatively few studies have addressed the properties of repressors. By studying the mechanisms by which MDM2 is capable of inhibiting p53-dependent transcription, we hope not only to understand the regulation of an important player involved in controlling cell growth but also to gain a more general insight into the functioning of transcriptional repressors.

To address the question of whether MDM2 can inhibit transcription in a p53-independent manner, we fused portions of the MDM2 protein to a heterologous DNA-binding domain to allow promoter recruitment in the absence of p53 binding. The ability of these proteins to repress transcription in both a p53-dependent and -independent manner was then tested using an MDM2-responsive, in vitro transcription system. In this way, we were able to demonstrate that MDM2 contains an inhibitory domain capable of repressing both basal and activated transcription. Furthermore, using an in vitro transcription system composed of recombinant proteins and highly purified HeLa cell nuclear fractions, we were able to identify the basal factors potentially involved in MDM2-mediated inhibition. In vitro binding assays designed to test the ability of MDM2 to interact with a minimal set of basal factors revealed a direct interaction between MDM2 and two components of the basal transcription machinery. Taken together, our data suggest that MDM2 may inhibit transcription from p53-responsive promoters by two distinct mechanisms: concealment of the activation domain of p53 and direct inhibition of the basal transcription machinery.

Results

Development and in vitro transcription system responsive to MDM2

To dissect the mechanism by which MDM2 inhibits p53-dependent transcription, we developed an MDM2-responsive, in vitro transcription system in which to study this process. MDM2 has not been demonstrated previously to function in in vitro transcription assays, possibly because of difficulties in obtaining purified, functional, recombinant MDM2. Human MDM2 is 491 amino acids in length and is poorly expressed and largely insoluble in both SF9 cells infected with recombinant baculoviruses and Escherichia coli (C. Thut and R. Tjian, unpubl.). In an attempt to circumvent the expression and solubility problems, we used a truncated version of MDM2 consisting of amino acid residues 1–324 (MDM2[1–324]) that had been shown previously to bind p53 (Brown et al. 1993). MDM2[1–324] was expressed and purified from E. coli by extracting proteins from lysed cell pellets under denaturing conditions. After purification and renaturation, the proteins were tested for their ability to inhibit in vitro transcription in a manner that mimics the specificity of MDM2 in transfection.
studies. Because MDM2 binds to the activation domain of p53 and requires this binding for its inhibitory effects, we reasoned that a hybrid protein containing the p53 activation domain fused to the Gal4 DNA-binding domain (G4-p53) would be sufficient for our study of MDM2 repression in vitro. We performed in vitro transcription reactions using a fractionated HeLa cell transcription system (Dignam et al. 1983) supplemented with purified, recombinant human TFIIA (Ranish et al. 1992) in place of the crude TFIIA-containing fraction. As expected, a template containing five Gal4 DNA-binding sites directs efficient transcriptional activation (approximately eightfold) in the presence of the G4-p53 protein (Fig. 1A, lanes 2,3). Addition of MDM2(1–324) to reactions containing G4-p53 resulted in up to a sixfold decrease in the level of transcription (Fig. 1A, lanes 4,5). Thus, our purified, renatured MDM2 protein was capable of inhibiting transcription dependent on the p53 activation domain. Next, we tested the specificity of the purified MDM2 by assaying its repressive effects on both

Figure 1. Development of an in vitro transcription system responsive to recombinant, human MDM2. (A) The first 324 amino acids of MDM2 specifically inhibit transcription dependent on the p53 activation domain in vitro. A protein containing the first 324 amino acids of human MDM2 was expressed and purified from bacteria and tested for its ability to inhibit transactivation by G4-p53, a protein consisting of the Gal4 DNA-binding domain (amino acids 1–94) fused to tandem copies of the p53 activation domain (amino acids 1–42). These proteins were assayed in an in vitro transcription system consisting of partially purified HeLa cell nuclear fractions and recombinant, purified hTFIIA using a template containing five Gal4-binding sites upstream the AdMLP. The promoter was fused to a G-less reporter, and the production of transcripts was measured by a G-less protocol. (Lane 1) The level of transcription in the absence of added activator; (lanes 2,3) reactions containing 30 ng of G4-p53. The reactions in lanes 4 and 5 contained both 30 ng of G4–p53 and 200 or 800 ng of purified, refolded MDM2(1–324), respectively. MDM2(1–324) was also tested for its ability to inhibit both basal transcription and transcription stimulated by the activation domain of VP16. (Lanes 7–10) 100 ng of a fusion protein consisting of the Gal4 DNA-binding domain (amino acids 1–147) and the activation domain of VP16 (amino acids 412–490). MDM2(1–324) protein was added to transcription reactions as follows, 200 ng (lanes 9,12), 400 ng (lane 13), or 800 ng (lanes 10,14). (B) The first 49 amino acids of MDM2 are required for an interaction with the activation domain of p53. GST (lanes 2,5) and a GST fusion protein containing amino acids 1–73 of p53 (lanes 2,5) were immobilized on glutathione resins and tested for their ability to retain soluble MDM2(1–324) (lanes 2,3) or an MDM2 protein lacking the first 49 amino acids [MDM2(50–324)] (lanes 5,6). After incubation with extracts containing the soluble MDM2 proteins, the resin and associated proteins were washed extensively, analyzed by SDS-PAGE, transferred to nitrocellulose, probed with monoclonal antibodies against MDM2 (Pharmagenics), and detected using an ECL protocol (Amersham). Lanes 1 and 4 represent 20% of the amount of MDM2 protein added to the corresponding binding reactions. (C) MDM2(50–324), which cannot interact with p53, is unable to inhibit G4–p53 dependent transcription in vitro. Using the transcription system described in A, MDM2(1–324) and MDM2(50–324) were tested for their ability to inhibit G4–p53 transactivation. Lanes 1 and 5 contain no added activator; lanes 2 and 6 contain 30 ng of G4–p53. In addition to 30 ng of G4–p53, 60 and 200 ng of MDM2(1–324) and 60 and 200 ng MDM2(50–324) were added to lanes 3 and 4 and lanes 7 and 8, respectively.
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G4–VP16-mediated and basal transcription. Previous studies performed in yeast had shown that p53 transactivation was inhibited by MDM2 but VP16 transactivation was not (Oliner et al. 1993). Likewise, in our in vitro system, transcriptional stimulation by G4–VP16 (Fig. 1A, lanes 7,8) was not inhibited by the addition of MDM2(1–324) (Fig. 1A, lanes 9,10). In addition, MDM2(1–324) did not inhibit basal transcription (Fig. 1A, lanes 11–14). These results, in combination, suggest that our simplified in vitro transcription system recapitulates the effects of MDM2 described previously in vivo.

Next, we wanted to determine whether an interaction between MDM2 and the activation domain of p53 was required for repression in vitro. Prior studies indicate that the first 49 amino acids of MDM2 are required for both its ability to bind p53 and its ability to inhibit p53-dependent transcription (Haines et al. 1994). We expressed and purified a truncated MDM2 protein containing amino acid residues 50–324 [MDM2(50–324)] from E. coli, as described above, and tested its ability to function in our transcription system. As shown in Figure 1B, this protein was no longer capable of repressing the interaction of p53 as assessed by in vitro binding assays. More importantly, MDM2(50–324) was no longer able to inhibit transcription by G4–p53 (Fig. 1C, lanes 5–8) at concentrations equivalent to those at which MDM2(1–324) could efficiently repress G4–p53 transcription (Fig. 1C, lanes 1–4). Thus, the interaction between MDM2 and p53 is important for inhibition of transcription in vitro as well as in vivo.

As mentioned previously, two models for MDM2’s mechanism of inhibition can be envisioned, both of which are consistent with the requirement for a direct, physical interaction between p53 and MDM2. First, MDM2 may conceal the activation domain of p53 through a direct interaction with this protein, thereby inhibiting basal factor contacts required for transcriptional stimulation. Second, MDM2 may contain an inhibitory domain capable of directly repressing the functions of one or more components of the basal machinery after being recruited to the promoter through an interaction with p53. If MDM2 represses transcription by the latter mechanism, it would be expected that MDM2 could also function as a repressor if targeted to a promoter via a p53-independent mechanism.

We wished to determine whether the MDM2/p53 interaction was required for MDM2 to inhibit p53-dependent transcription. To address this question, we asked whether MDM2(50–324) could inhibit the p53 activation domain when recruited to a promoter by fusion with a heterologous DNA-binding domain. A template containing five Gal4 sites upstream of two LexA sites was used to target both an MDM2/Gal4 fusion protein [G4–MDM2(50–324)] and Lex–p53 (the LexA DNA-binding domain fused to two copies of the activation domain of p53) to the E4 promoter. Lex–p53 is able to stimulate transcription from this template, as shown in Figure 2 (lanes 2,6). By adding increasing concentrations of G4–MDM2(50–324) to reactions containing Lex–p53, we were able to demonstrate that MDM2(50–324) was capable of inhibiting p53-dependent transcription once its ability to be recruited to the promoter was restored (Fig. 2, lanes 3–5). Addition of the Gal4 DNA-binding domain itself does not repress Lex–p53-dependent transcription (Fig. 2, lanes 8,9). Thus, it does not appear that simple occupancy of the Gal4 sites precludes binding of Lex–p53.
and subsequent activation. Furthermore, this inhibitory effect is not attributable to a direct recruitment of G4-MDM2(50–324) by Lex-p53 because G4-MDM2(50–324) does not inhibit transcription from a template lacking Gal4-binding sites (Fig. 2, lanes 12–14). Taken together, these results demonstrate that recruitment of MDM2 to a template is sufficient to allow inhibition of p53-dependent activation.

MDM2 contains a domain that can repress basal transcription

Because of the close proximity of G4-MDM2 and Lex-p53 when bound at the promoter, it was possible that G4-MDM2(50–324), although incapable of efficient in vitro binding, was still weakly interacting with the activation domain of p53 and thereby inhibiting its ability to activate transcription. For this reason, we were interested in determining whether MDM2 possessed a p53-independent inhibitory domain that could repress basal transcription directly. To this end, we tested the activity of G4-MDM2(50–324) in a fractionated HeLa cell transcription reaction. For these assays, two templates were added to each transcription reaction; one template contained five Gal4-binding sites upstream of the adenovirus major late promoter (AdMLP) to measure G4-MDM2 inhibition of transcription, and one contained only the AdMLP to serve as an internal negative control. Transcript production was assayed using a G-less protocol, and radioactive products arising from the two different templates were differentiated based on the size of their products. In this system, G4-MDM2(50–324) was capable of inhibiting basal transcription in a Gal4 site-dependent manner (Fig. 3, lanes 1–5; three- to fourfold inhibition). The G4 DNA-binding domain alone did not inhibit basal transcription in this system (data not shown). These results are consistent with the presence of an MDM2 domain capable of directly inhibiting one or more components of the basal machinery.

TFIIA and TAFs are not required for MDM2 to inhibit basal transcription

Because MDM2 was able to repress basal transcription, we thought it likely that MDM2 was directly inhibiting the functions of one or more of the general transcription factors. To narrow the search for the basal factor component(s) inhibited by MDM2, we tested the ability of MDM2 to repress reconstituted basal transcription reactions lacking several of the general transcription factors. In the absence of an activator, it has been demonstrated that both TFIIA and the TAF [TATA-binding protein (TBP)-associated factor] components of the TFIIID complex are dispensable for accurately initiated transcription from promoters containing TATA boxes. We therefore tested the ability of G4-MDM2(50–324) to inhibit transcription in a reconstituted, in vitro reaction containing recombinant TBP, TFIIA, TFIIF, and TFIIIE and highly purified polymerase II (Pol II) and TFIIH. This complement of general transcription factors was capable of supporting transcription in the dual template system described previously (Fig. 3, lane 10). The purified system appears to allow transcription from an additional start site from each template, as evidenced by the appearance of a closely spaced doublet of bands. This phenomenon is likely to be the result of substituting TBP for TFIIID (C. Thut and R. Tjian, unpubl.). The presence of this additional start site, however, did not affect the interpretation of the results. In this system, G4-MDM2(50–324) retained its ability to repress basal transcription (Fig. 3, lanes 6–9; upper transcript, three- to fourfold inhibition). Furthermore, this repression was dependent on the presence of Gal4 sites in the promoter (Fig. 3, lanes 6–9, lower transcript). Again, the Gal4 DNA-binding domain alone did not repress transcription (data not shown). The ability of G4-MDM2 to inhibit transcription in the absence of TFIIA and TAFs suggests that one of the remaining basal factors may be the target of the repressive activity of MDM. In addition, the purity of this MDM2-responsive transcription system argues against a mechanism of MDM2 action in which MDM2 recruits a
corepressor or additional factor to mediate the observed repression.

The inhibitory domain of MDM2 lies between amino acid residues 50 and 222 and can repress p53-dependent transcription in vivo.

If MDM2 does target one of the basal factors, it might be expected that MDM2 would directly interact with this factor. However, before testing the ability of MDM2 to bind the general transcription factors, we chose to more narrowly define the region of MDM2 containing the basal inhibitory domain. By this method, we hoped to limit the number of observed interactions unrelated to the inhibitory mechanism. To this end, 276 amino acids were removed from the carboxyl terminus of MDM2, and the remaining MDM2 sequences were fused to the DNA binding domain of Gal4 (G4–MDM2 1–324). Like G4–MDM2 (1–324) and G4–MDM2 (50–324), G4–MDM2 222 was capable of inhibiting both p53-activated transcription (data not shown) and basal transcription (Fig. 4, lanes 6–9). Together, these results suggest that the basal inhibitory domain of MDM2 lies between amino acids 50 and 222.

To this point, we had identified a domain of MDM2 that was capable of inhibiting both basal and activated transcription in vitro. To determine whether this inhibitory domain was also important for the ability of MDM2 to repress transcription in vivo, we asked whether a fusion protein containing the Gal4 DNA-binding domain and amino acids 50–222 of MDM2 could inhibit full-length p53 in transiently transfected C-33A cells (a human cervical carcinoma cell line devoid of p53). In these experiments, transfection of an expression construct encoding full-length p53 resulted in a 30- to 60-fold enhancement in transcription from a template containing four p53 sites upstream of five Gal4 sites and the E1B TATA box. This promoter was fused upstream of the chloramphenicol acetyltransferase (CAT) gene, allowing promoter activity to be measured by assaying transfected cell extracts for CAT activity. Figure 4B summarizes the results of three sets of transfection experiments in which transcriptional stimulation in the presence of p53 is normalized to 1. In these experiments, cotransfection of a G4–MDM2 (50–222) expression vector with the p53 expression vector and the p53/Gal4 site template significantly reduced the levels of CAT activity measured. At the highest concentration of transfected G4–MDM2 (50–222) vector, the promoter activity is reduced to 5% that of p53 alone (Fig. 4B).

This transcriptional inhibition is not attributable to simple occupancy of the Gal4 sites between the p53 sites and the start of transcription because expression of the Gal4 DNA-binding domain alone does not repress p53-dependent transcription (Fig. 4B), though G4 and G4–MDM2 (50–222) are expressed at similar levels (data not shown). Instead, transfection of the Gal4 DNA-binding domain is slightly stimulatory, which is not unexpected as this protein has been suggested previously to contain a cryptic activation domain. Furthermore, the repression of transcription from this promoter...
is not attributable to a general repression of transcription resulting from G4-DM2 overexpression because the inhibition is dependent on the presence of Gal4 sites in the template (Fig. 4B). These results demonstrate that the inhibitory domain of MDM2 identified in vitro is also capable of repressing transcription directed by full-length p53. In addition, these findings suggest that the putative repressor domain of MDM2 is capable of inhibiting transcription in a cellular setting in which the templates are likely to be assembled into chromatin and additional proteins that might interact with either p53 or MDM2 are present.

The inhibitory domain of MDM2 interacts with both the 34K subunit of TFIE and TBP

With a shortened version of the MDM2 protein containing amino acids 1-222 (MDM222), the ability of the inhibitory domain of MDM2 to bind components of the basal transcription machinery was tested. By fusing the first 222 amino acids of MDM2 to the glutathione S-transferase (GST) moiety and immobilizing this protein on a glutathione-Sepharose resin, we assessed the ability of the MDM2 inhibitory domain to interact selectively with soluble forms of the basal factors TBP, TFIIIB, TFIIIF, Pol II, and the 34K and 56K subunits of TFIE. Proteins that remained bound to the GST–MDM222 resins after extensive washing were separated by SDS-PAGE, blotted to nitrocellulose, and detected using antibodies specific for these factors. As a positive control, the G4-p53 activation domain fusion was also tested for its ability to bind these resins (Fig. 5A, lanes 19–21). As shown in Figure 5A, TBP (lanes 10–12) and the 34K subunit of TFIE (lanes 13–15) were the only basal factors that were retained to a significant extent by the GST–MDM222 beads but not the GST resin. The observed interaction between MDM2 and TBP was not unexpected and has been reported previously (Leng et al. 1995). The TFIE 56K subunit was also retained to a small extent (Fig. 5A, lanes 16–18; <5% of the input bound), though binding to the GST resin lacking MDM2 was also detected, making it difficult to determine definitively whether TFIE 56K and MDM2 interact specifically.

Because TBP is notoriously “sticky” and has been reported to bind a large number of proteins, we wanted to obtain additional evidence that the interaction between TBP and MDM2 could occur in vivo. In a cellular setting, the majority of TBP is thought to exist in large multiprotein complexes, including TFIIID, SL1, and TFIIIB (Goodrich and Tjian 1994a and references therein). For this reason, we tested the ability of MDM222 to interact with the TFIIID complex. We linked antibodies against the TAFn$_{130}$ subunit of TFIIID to protein A–Sepharose beads and immobilized the TFIIID complex from partially purified HeLa extracts on this resin. Next, extracts containing bacterially expressed MDM222 were tested for their ability to interact with this immobilized complex. Unlike the monomeric form of TBP, TBP incorporated into the TFIIID complex was not able to interact with MDM222 (Fig. 5B, lanes 1–3). This finding suggests that an interaction between MDM2 and TBP may not occur in vivo and thus this interaction may not be relevant to the inhibitory properties of MDM2.

Discussion

In this report we provide evidence that MDM2, an oncoprotein that inhibits p53-dependent transcription, contains a previously undetected repression domain between amino acids 50 and 222 that can inhibit basal transcription in a p53-independent manner. Several mechanisms have been proposed to explain the ability of repressors to inhibit basal transcription, including direct inhibition of the activities of the general transcription machinery by the repressor (Sauer et al. 1995), modification of chromatin structure near the promoter (Bunker and Kingston 1994), and recruitment of additional factors or “corepressors” that themselves mediate transcriptional inhibition (Keleher et al. 1992; Paroush et al. 1994). We have demonstrated that MDM2 is able to inhibit basal transcription in a highly purified system devoid of chromatin. Furthermore, this system is not likely to contain potential corepressors, unless they are tightly associated with either Pol II or TFIIH. Our studies of the repression domain of MDM2 thus suggest that this domain directly inhibits one or more functions of the basal machinery. In an attempt to identify components of the basal machinery that might be targeted by MDM2, we used a combination of in vitro transcription assays and in vitro binding assays to define a subset of the basal factors sufficient to support MDM2-dependent basal inhibition and to identify potential contacts between MDM2 and the general transcription machinery. These studies led to the finding that neither TFIIA nor the TAFs are necessary for the ability of MDM2 to inhibit transcription. Of the remaining basal factors, MDM2 was found to interact with both the 34K subunit of TFIE and monomeric TBP, suggesting that these contacts MDM2 might inhibit their ability to function in the preinitiation complex. However, the additional finding that the inhibitory domain of MDM2 was not able to interact with TBP when in complex with TAFs leads us to speculate that the interaction between MDM2 and TBP may not be functionally relevant.

Our finding that the 34K subunit of TFIE may be the target of the repressive activities of MDM2 was intriguing because it has been shown previously that a Drosophila repressor, Krüppel, also directly interacts with the 34K subunit of TFIE (Sauer et al. 1995). During transcription initiation, TFIE is thought to play a key role in the ability of the general transcription machinery to progress from the promoter-bound complex to the actively elongating complex, and repressors that target TFIE might disrupt this transition by inhibiting one or more of the known functions of TFIE. TFIE appears to work in concert with the basal transcription factor TFIIH to facilitate DNA strand separation at the promoter, leading to promoter clearance and elongation (Schaef er et al. 1993; Goodrich and Tjian 1994a; Holstege et al. 1995, 1996). TFIE is also believed to be required for the
recruitment of TFIIH (Flores et al. 1992; Maxon et al. 1994), a multisubunit complex that contains several enzymatic activities itself, including helicase, kinase, and ATPase (Sopta et al. 1989; Flores et al. 1992; Schaeffer et al. 1993; Serizawa et al. 1993; Roy et al. 1994). These enzymatic properties of TFIIH have been shown to be regulated in vitro by TFIIE (Lu et al. 1992; Ohkuma and Roeder 1994; Serizawa et al. 1994; Ohkuma et al. 1995). By binding to the small subunit of TFIIE, it is possible that MDM2 affects the ability of TFIIH to recruit TFIIH or to influence its kinase, helicase, or ATPase activities.

Alternatively, TFIIE may play a more direct role in DNA strand separation or recruitment and stability of the pre-initiation complex, offering other potential functions to be targeted by MDM2 (Maxon et al. 1994; Holstege et al. 1995). Future studies will address the ability of MDM2 to inhibit these specific functions of TFIIH in an attempt to determine the mechanism by which MDM2 can directly repress basal transcription.

Although the precise mechanisms by which specific
repressors exert their inhibitory activities are not well
known, at least three types of repressors have been pro-
posed to exist: those that compete with activators for
promoter binding sites, “quenchers” that directly inhibit
the action of specific transactivators, and inhibitors that
directly repress the functioning of the basal transcription
machinery (Levine and Manley 1989; Johnson 1995).

Much circumstantial evidence has been accumulated to
suggest that MDM2 represses the transactivating func-
tions of p53 by masking or interfering with its activation
domain. This domain of p53 has been shown to interact
with several components of the transcription machinery,
including TAFII32 (Lu and Levine 1995; Thut et al. 1995),
TAFII70 (Thut et al. 1995), TBP (Seto et al. 1992; Chen et
al. 1993; Liu et al. 1993; Truant et al. 1993), and TFIIH
(Xiao et al. 1994). A functional importance for these in-
teractions has been shown in the case of TAFII32 and
TAFII70 (Thut et al. 1995). Interestingly, the same mu-
tation in the activation domain of p53 that impairs its
ability to bind TAFII32 (Lu and Levine 1995; Thut et al.
1995), TAFII70 (Thut et al. 1995), and TBP (amino acids
22 and 23) also disrupts its ability to contact MDM2 (Lin
et al. 1994), suggesting that MDM2 and the basal factors
may interact with overlapping portions of p53. Furth-
ernore, the recent crystal structure of the p53/MDM2 in-
teraction interface showed that residues 22 and 23 are
contained within this interface. These correlations lend
further support to the hypothesis that MDM2 inhibits
p53-dependent activation by disrupting interactions
with components of the general transcription machin-
ery.

Our results suggest that MDM2 can also directly in-
hit the ability of the general transcription factors to
potentiate mRNA synthesis. However, we do not believe
that our findings exclude the possibility that MDM2 may
also inhibit p53-dependent transcription by mask-
ing its activation domain from important basal factor
contacts. On the contrary, the experiments described
here, in conjunction with the correlation between the
MDM2 and basal factor binding sites in p53's activation
domain, suggest that it is likely that MDM2 functions as
both a quencher of p53 and a direct inhibitor of the basal
machinery (Fig. 6). In addition, it is possible that these
two mechanisms of inhibition by MDM2 complement
each other and enhance the ability of MDM2 to regulate
p53-responsive promoters. In support of this hypothesis,
a similar dual mechanism of inhibition involving both
potential masking of an activator and recruitment of a
general repression domain has also been proposed to ex-
plain the inhibition of E2F-dependent transcription by
Rb (Bremner et al. 1995; Sellers et al. 1995; Weintraub et
al. 1995). Much like the MDM2/p53 case, an interaction
between the activation domain of E2F and Rb has been
shown to be important for the ability of Rb to inhibit
transcription (Flemington et al. 1993; Hagemeier et al.
1993; Helin et al. 1993). In addition, Rb is capable of
repressing transcription in an E2F-independent manner
when fused to a heterologous DNA-binding domain,
although it is not clear whether Rb targets the basal ma-
achinery or whether it serves to quench nearby activators
(Bremner et al. 1995; Sellers et al. 1995; Weintraub et al.
1995). These results suggest that transcriptional inhibi-
tors that repress transcription via multiple, distinct
mechanisms are not so uncommon.

If MDM2 can inhibit the transactivating ability of p53
by directly interacting with this protein, why then
should MDM2 contain an additional inhibitory domain?
We believe that the answer to this question is best ad-
ressed in the context of complex eukaryotic promoters.
Differential gene expression, especially in metazoans, is
regulated by integrating a wide variety of positive and
negative growth signals that dictate the level of tran-
scripts arising from a particular promoter. This integra-
tion is likely to occur by the cooperative action of mul-
tiple transcriptional regulators bound at a promoter,
each of which can influence the functions of the basal
transcription machinery. Because most complex regula-
tory elements bear multiple activator binding sites, it is
expected that efficient inhibition of transcription from
such a promoter would require either multiple repressors
affecting different sets of activators or a single repressor
or silencer capable of impeding transactivation of mul-


Figure 6. A dual mechanism for inhibition of p53-responsive
promoters by MDM2. The current model for inhibition of p53-
dependent transcription by MDM2 suggests that by binding to
the activation domain of p53, MDM2 disrupts important func-
tional interactions between this domain and components of
the basal transcription machinery. Our data suggest that MDM2
also contains a p53-independent inhibitory domain that directly
represses basal transcription. The observed interaction be-
 tween the inhibitory domain of MDM2 and the 34K subunit of
the basal factor TFII E may indicate that MDM2 represses the func-
tion of this basal factor and thereby inhibits the ability of the
preinitiation complex to synthesize mRNA. We propose that
these inhibitory mechanisms are not mutually exclusive and
may play different roles in the repressive properties of MDM2.
Perhaps MDM2 simultaneously conceals the activation domain
of p53 and directly represses the function of one or more com-
ponents of the basal machinery to more stringently control tran-
scription from p53-responsive promoters.

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Mammalian expression constructs for Gal4(1–94) and Gal4–MDM2(250–222) were constructed by digesting the NdeI–EcoRI fragment from the corresponding His–MDM2 vectors into a pET 3a-based vector containing DNA sequences encoding the first 94 amino acids of the Gal4 DNA-binding domain (J. Goodrich, unpubl.) cut with KpnI (blunt) and EcoRI. To produce G4–MDM2(250–222), the Ndel and EcoRI sites were created on either side of the MDM2 coding sequences by PCR, and digested with Ndel and EcoRI and ligated into Ndel/EcoRI-cut Gal4 vector. GST–MDM2(1–222) was constructed by digesting His–MDM2(1–222) with Ndel and EcoRI and ligating this fragment into Ndel/EcoRI-cut pGEX–2TK (S. Ruppert, unpubl.). The (p53(Gal4)E18BCAT) transcription template used in transfections was produced by inserting four p53 sites contained on a HindIII–PstI fragment from pc2 PG4-lacZ (a kind gift from B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) into HindIII–PstI-cut G4–E18BCAT (Lillie and Green 1989). Mammalian expression constructs for Gal4(1–94) and Gal4–MDM2(250–222) were constructed by transferring the Ndel–EcoRI fragments from the E. coli expression vectors containing G4 and G4–MDM2(250–222) into a derivative of pTRIP (S. Ruppert, unpubl.) to insert a Kozak sequence (Ncol site) upstream of the coding sequences. Next, a HindIII–EcoRI fragment containing the Kozak sequence and the coding regions was inserted into pcDNA3.1+ (Invitrogen) to yield pcDNA–G4 and pcDNA–G4–MDM2(250–222).

Purification of recombinant proteins

The MDM2(1–324) and MDM2(250–324) proteins were expressed in E. coli (BL21 strain) by transfecting bacteria with His–MDM2(1–324) and His–MDM2(250–324). The cells were grown to an OD600 of 0.5 and induced using 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by an additional 4 hr of growth at 37°C. The cells were pelleted and sonicated, and the lysates were cleared by centrifugation. The cell pellets were then washed with urea buffer (5 mM urea, 10 mM MES at pH 6.0, 10 mM ZnSO4, 10 mM β-mercaptoethanol [β-me] and 0.1 M NaCl) containing 35 mM imidazole. The proteins were eluted from the resin using urea buffer containing 250 mM imidazole and loaded on a Poros HS column (PerSeptive Biosystems). The Poros HS column was eluted using a 0.1–1.0 M NaCl gradient in urea buffer, and fractions containing His-MDM2, as assessed by SDS-PAGE and Coomassie staining, were pooled. The denatured proteins were then refolded by stepwise dialysis first into guanidine buffer then into non-denaturing buffer (20 mM Tris at pH 7.9, 10 mM ZnSO4, 10 mM β-me, 0.1 M NaCl) containing 35 mM imidazole. The proteins were then washed with urea buffer (5 mM urea, 10 mM MES at pH 6.0, 10 mM ZnSO4, 10 mM β-me, 0.1 M NaCl) containing 35 mM imidazole and loaded onto a Poros HS column (PerSeptive Biosystems). The Poros HS column was eluted using a 0.1–1.0 M NaCl gradient in urea buffer, and fractions containing His-MDM2, as assessed by SDS-PAGE and Coomassie staining, were pooled. The denatured proteins were then refolded by stepwise dialysis first into guanidine buffer then into non-denaturing buffer (20 mM Tris at pH 7.9, 10 mM ZnSO4, 10% glycerol) using the steps 2 M guanidine, 1 M guanidine, 0.5 M guanidine, and 0.1 M KCl.

Materials and methods

**Plasmids**

E. coli expression vectors containing human MDM2 sequences corresponding to amino acids 1–324, 50–324, and 1–222 were constructed using a PCR-based strategy. Ndel sites were placed at the first or fifteenth codon of human MDM2, and a stop codon followed by a BamHI site was placed after amino acid 222 or 324. The PCR products and pET19b (Novagen) were then digested with Ndel and BamHI and ligated to produce the His-MDM2(1–324) and His-MDM2(250–324) vectors. His–MDM2(1–222) was constructed by placing an Ndel site at the first codon and a stop codon followed by an EspI site after amino acid 222, digesting the PCR product and pET19b with Ndel and EspI and ligationing the fragments. G4–MDM2(1–324), G4–MDM2(250–324), and G4–MDM2(1–222) were engineered by ligating an Ndel (blunt)/EcoRI fragment from the corresponding His-MDM2 vectors into a pET 3a-based vector containing DNA sequences encoding the first 94 amino acids of the Gal4 DNA-binding domain (J. Goodrich, unpubl.) cut with KpnI (blunt) and EcoRI. To produce G4–MDM2(250–222), the Ndel and EcoRI sites were created on either side of the MDM2 coding sequences by PCR, and digested with Ndel and EcoRI and ligated into Ndel/EcoRI-cut Gal4 vector. GST–MDM2(1–222) was constructed by digesting His–MDM2(1–222) with Ndel and EcoRI and ligationing this fragment into Ndel/EcoRI-cut pGEX–2TK (S. Ruppert, unpubl.). The (p53(Gal4)E18BCAT) transcription template used in transfections was produced by inserting four p53 sites contained on a HindIII–PstI fragment from pc2 PG4-lacZ (a kind gift from B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) into HindIII–PstI-cut G4–E18BCAT (Lillie and Green 1989). Mammalian expression constructs for Gal4(1–94) and Gal4–MDM2(250–222) were constructed by transferring the Ndel–EcoRI fragments from the E. coli expression vectors containing G4 and G4–MDM2(250–222) into a derivative of pTRIP (S. Ruppert, unpubl.) to insert a Kozak sequence (Ncol site) upstream of the coding sequences. Next, a HindIII–EcoRI fragment containing the Kozak sequence and the coding regions was inserted into pcDNA3.1+ (Invitrogen) to yield pcDNA–G4 and pcDNA–G4–MDM2(250–222).

**Purification of recombinant proteins**

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**G4–MDM2(1–324), G4–MDM2(250–324), and G4–MDM2(1–222) were expressed and purified by a protocol similar to that for the His-MDM2 proteins. However, after extraction from the...**
pellet, the Ni\textsuperscript{2+} affinity chromatography step was omitted and the denatured proteins were dialyzed into urea buffer and purified using a Poros HS column. The resulting protein pools were then dialyzed back into guanidine buffer and purified by size-exclusion chromatography using a Superose 12 column (Pharmacia). Renaturation followed the same procedure as that of the His–MDM2 proteins.

Lex-p53 was expressed by transforming the Lex-p53 plasmid into E. coli (BL21 strain), growing the cells to an OD\textsubscript{600} of 0.5, inducing with 0.4 mM IPTG for 4 hr, and collecting the cells. The resulting cell pellet was resuspended in TEGM (20 mM Tris at pH 7.9, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, 20% glycerol) containing 0.1 M NaCl and 0.1% NP-40, and sonicated, and the lysates were cleared by centrifugation. Lysates were then applied to anti-Flag M2 antibody resin (Kodak), nutated at 4°C for 4 hr, and eluted using Flag epitope peptide dissolved in TEGM containing 0.1 M NaCl and 0.1% NP-40.

G4-p53 was expressed as described previously (Thut et al. 1995) and purified using DEAE CL4B (Pharmacia) and Poros HS resins (PerSeptive Biosystems) eluted with linear salt gradients. GST–p53 and GST–MDM2(1–222) were expressed in BL21 cells transformed with the appropriate plasmids. Crude lysates from these cells were immobilized on glutathione–Sepharose beads (Pharmacia) by incubating the resin and cell extracts for 1 hr and washing the resin with TEGM/0.1 M NaCl. The beads were equilibrated to TEGM/0.1 M NaCl before they were used in in vitro binding assays. G4-VP16 was expressed and purified as described previously (Chasman et al. 1989).

In vitro binding assays
In vitro protein–protein interaction assays using GST fusion proteins were performed essentially as described (Goodrich et al. 1993). Approximately 0.5–1 µg of the immobilized proteins and 200–400 ng of the soluble basal factors were used in each binding reaction. The interaction assay using TFIIID complexes immobilized on beads was performed by binding monoclonal antibodies against hTAF\textsubscript{II},130 to protein A–Sepharose resin (Pierce) in TEGM/0.4 M NaCl for 1 hr, washing the unbound antibodies away with TEGM/1.0 M NaCl + 0.1% NP-40, and incubating a HeLa nuclear P1.0 fraction (Dignam et al. 1983) with the antibody beads overnight at 4°C. The beads were then washed three times with TEGM + 0.7 M NaCl and three times with TEGM + 0.1 M NaCl. The immobilized TFIIID complexes were assayed for their ability to bind soluble extracts containing MDM(1–222) by a procedure similar to that used for the GST fusion protein/basal factor interactions.

Transient transfection assays
C33A cells (ATCC) were plated at a density of 3 \times 10^5 cells per well in six-well plates (Falcon) 16–24 hr before CaPO\textsubscript{4}-mediated transfection (Sambrook et al. 1989). The medium (Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 U/ml of streptomycin, and 2 mM glutamine) was replaced 18–24 hours after transfection. Twenty-four to 36 hr later, the cells were harvested, lysed, and assayed for β-galactosidase and CAT activity (Sambrook et al. 1989).

Acknowledgments
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