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Identification of a Domain Within MDMX-S That is Responsible for its High Affinity Interaction With p53 and High-Level Expression in Mammalian Cells

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Abstract The MDMX gene product is related to the MDM2 oncoprotein, both of which interact with the p53 tumor suppressor. A novel transcript of the *MDMX* gene has been previously identified that has a short internal deletion of 68 base pairs, producing a shift in the reading frame after codon 114, resulting in the inclusion of 13 novel amino acids (after residue 114) followed by a stop codon at amino acid residue 127. This truncated MDMX protein, termed MDMX-S, represents only the p53 binding domain and binds and inactivates p53 better than full-length MDMX or MDM2. Here we show that when expressed in cells, MDMX-S is targeted more efficiently to the nucleus than MDMX. MDMX-S suppresses p53-mediated transcription from a p53 target promoter better than full-length MDMX. The DNA damage inducibility of these p53 responsive promoters was suppressed better by MDMX-S than by MDMX. Analysis of the MDMX-S protein indicated that the 13 novel amino acids at its carboxy terminus was responsible for high affinity binding to p53 in vitro and for high level expression of the protein in cells. Deletion of this 13 amino acid sequence resulted in a protein that was not able to bind p53 and was not able to be expressed well in cells. Taken together, these data point to an important domain within MDMX-S that enables it to function well in vivo to block p53 activity. J. Cell. Biochem. 89: 563–575, 2003.
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The MDM2 oncoprotein appears to play an important role in neoplastic transformation by interacting with a number of cell cycle regulatory proteins within the cell. One of the best characteristics of these MDM2-interactive factors is the p53 tumor suppressor protein [Mom and Zambetti, 1997; Piette et al., 1997; Freedman et al., 1999]. Through this interaction, MDM2 inactivates p53. MDM2 accomplishes this function by blocking the ability of p53 to activate transcription, by targeting p53 for proteolytic breakdown and by transporting p53 out of the nucleus into the cytoplasm [Oliner et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997; Thut et al., 1997; Roth et al., 1998]. Given these facts regarding MDM2, it would therefore be expected that other factors in the cell with functions similar to MDM2 could play an important role in the deregulation of cell cycle control and apoptosis via p53 inactivation.

A protein termed MDMX has been cloned that is structurally and functionally related to MDM2 [Shvarts et al., 1996, 1997]. MDMX and MDM2 are nearly identical in size, yet only share some common domains. For example, MDMX and MDM2 are similar within the p53-binding domain at their amino termini and both contain a central zinc-finger domain and a carboxy terminal ring-finger domain [Boddy and Freemont, 1994; Shvarts et al., 1996, 1997]. Like MDM2, MDMX has been found to associate with and suppress the activity of p53 [Shvarts et al., 1996]. Additionally it has been shown

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that both MDM2 and MDMX bind the p53 related factors p63 and p73 [Balint et al., 1999; Ongkeko et al., 1999; Kadakia et al., 2001]. Like their effects on p53, both MDMX and MDM2 were able to block the transactivation function of both isoforms of p63 but only MDM2 was able to target p63 for proteolytic degradation [Kadakia et al., 2001]. However, in contrast to their effect on p53, MDMX and MDM2 stabilize p73 by protecting it from proteolytic degradation [Ongkeko et al., 1999]. In this way, the growth suppressive functions and inducibility of the *p21* gene by p73 were enhanced by MDMX and MDM2 [Ongkeko et al., 1999]. Interestingly, MDMX is located primarily in the cytoplasm [Rallapalli et al., 1999; Jackson and Berberich, 2000; Jackson et al., 2001], unlike MDM2 which is nucleolar [Li et al., 2002]. However, MDMX can be induced to translocate to the nucleus upon treatment of cells with DNA damaging agents [Li et al., 2002].

In addition to their ability to interact with p53 and p73, MDMX and MDM2 have been shown to heterodimerize, by interacting through their respective ring fingers at their carboxy termini [Sharp et al., 1999]. The formation of such a heterodimer appears to indirectly stabilize p53, due to an inability of MDM2 to bind p53 [Jackson and Berberich, 1999; Sharp et al., 1999]. However, it appears that MDM2 and MDMX are functionally dependent on one another in that MDMX stabilizes MDM2 so that the latter can block p53, p63, and p73 [Gu et al., 2002]. In this regard MDMX may therefore have functions that are distinct from that of MDM2, but yet enable MDMX to act as an oncoprotein as has been recently suggested [Migliorini et al., 2002]. However, evidence suggests that the only role of MDMX in control of cell proliferation and the cell cycle is through its regulation of p53 [Migliorini et al., 2002]

This latter finding is surprising given that MDMX interacts with all p53 family members and as been recently shown, MDMX interacts with the E2F1 transcription factor, reducing the ability of E2F1 to bind DNA [Strachan et al., 2003]. This interaction also leads to an E2F-1-mediated breakdown in the MDMX protein [Strachan et al., 2001]. Given that MDMX associates with E2F1, MDM2, p53, p63, and p73, it is therefore likely that MDMX will interact with additional cellular factors, which may extend its control over the cell cycle and apoptosis.

Another form of MDMX regulation is found in an alternatively spliced transcript, termed MDMX-S. MDMX-S has a small internal deletion that leads to the introduction of a stop codon following amino acid residue 127 [Rallapalli et al., 1999]. This novel transcript encodes only the p53-binding domain of MDMX with the addition of 13 novel amino acid residues. This protein produced has been referred to as MDMX-S (short form) [Rallapalli et al., 1999]. MDMX-S has been shown to be better able to suppress p53-mediated transcriptional transactivation and induction of apoptosis, relative to full-length MDMX or MDM2. MDMX-S expression appears to be upregulated in a variety of growing and transformed cell lines while it is expressed to very low levels in normal low passage nontransformed cells. With these facts in mind, MDMX-S may therefore play an important role in the regulation of tumorigenesis. Here we explore the basis by which MDMX-S binds p53. We have analyzed domains within the MDMX-S protein, to determine the regions responsible for this interaction. Our analysis reveals that the unique 13 amino acid region at the carboxy terminus of MDMX-S is needed for both a high affinity interaction with p53 and for high level protein expression in cells.

MATERIALS AND METHODS

Cell Culture, Plasmids, Transfections and Apoptosis

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum or fetal calf serum. All transfection experiments were initiated on 50% confluent monolayer cultures. Plasmids (a total of 30 μg) were transfected by the calcium phosphate procedure. The cells were washed the next day to remove the precipitate.

The FLAG epitope tagged MDMX and MDMX-S cDNA's were cloned into the eukaryotic expression vector pRC/CMV (InVitrogen). The wild-type p53 cDNA was cloned into and expressed from the pcDNA3 expression plasmid (InVitrogen). The pRGCDFosLacZ reporter construct containing multiple p53 consensus DNA binding sites cloned upstream of the basal Fos promoter was used as a p53-target promoter [Frebourg et al., 1992]. As a control, the pDFosLacZ reporter used is identical to pRGCDFosLacZ, but lacks the p53 consensus sequences [Frebourg et al., 1992].

Extracts were generated by multiple freeze thaw cycles approximately 24 h after the trasnfection. Equal amounts of protein from the soluble extracts were assayed either for CAT activity by thin layer chromatography and autoradiography or for beta-galactosidase activity by a color reaction. All transfections were performed multiple times. A plasmid containing the SV40 early promoter linked to luciferase was included in all transfections of the beta-galactosidase constructs so that the beta-gal activity could be normalized for differences in transfection efficiency.

Generation of Extracts and Fusion Proteins

Extracts for immunoblots were generated by lysing the cells on ice in 0.1% NP-40, 10 mM Tris (pH 7.9), 10 mM $MgCl_2$, 15 mM NaCl and the protease inhibitors PMSF (0.5 mM), pepstatin (2 μg/ml) and leupeptin (1 μg/ml). The soluble fraction was termed the cytosol. The nuclei were pelleted by centrifugation at 800*g* for 10 min and resuspended in extraction buffer consisting of 0.42 M NaCl, 20 mM HEPES (pH 7.9), 20% glycerol, PMSF (0.5 mM), pepstatin (2 μg/ml) and leupeptin (1 μg/ml) for 10 min on ice, then centrifuged at 14000*g* for 8 min to pellet the residual nuclear material. This supernatant fraction was termed nuclear extract [Moberg et al., 1992].

To generate GST fusion proteins, the MDMX and MDMX-S cDNAs were cloned into pGEX-5T (Pharmacia) in frame with the glutathione-S-transferase gene (*GST*). Fusion proteins were produced as described in Jordan et al. [1994]. Briefly, the genes were induced with IPTG (0.5 mM) for 3 h, bacteria were lysed, and protein was separated from cellular debris by centrifugation. The bacterial extracts were applied to small batch columns of glutathione sepharose (Pharmacia) to generate affinity colums containing the various fusion proteins [as in Jordan et al., 1994].

RNA Analysis and RT/PCR Reactions

Total RNA was isolated from cells by Trizol (Gibco/BRL). For RT/PCR reactions, 1 μg of total RNA was used in each reaction with primers from the murine sequence [the 5′ primer: gccctctctatgacatgc (spanning amino acid residues 96-102) and the 3′ primer: gtcgtgaggtaggcag (spanning amino acid residues 158-163)]. All total RNA's were DNAase I treated in the RT reaction prior to the PCR reactions. Additionally, as a control, PCR done in the absence of RT was negative for any ethidium bromide stained bands (data not shown).

In Vitro Transcription/Translation

In vitro transcription reactions were performed with Bluescript KS plasmids containing the MDMX and MDMX-S cDNAs. The plasmid constructs were used directly in the TnT coupled in vitro transcription/translation system of Promega, using the nuclease treated rabbit reticulocyte lysate, in a total reaction volume of 50 μl. ^{35}S-methionine at 0.9 μCi/ml was also included in the reactions.

Western Blot Hybridizations

Extracts were first electrophoresed by SDS-PAGE and then the proteins were electrophoretically transferred onto nitrocellulose, the blots washed in TBST buffer (10 mM Tris, pH 8; 150 mM NaCl; 0.05% Tween 20), blocked with 2.5% BSA in TBST for 30 min at room temperature and then incubated with the M2 anti-FLAG monoclonal antibody (10 ng/ml, VWR/Kodak/IBI). The blots were washed three times (10 min each) with TBST, then incubated for 30 min at room temperature with a 1:7500 dilution of secondary antibody in TBST (goat anti-mouse conjugated to alkaline phosphatase, Vector Labs). The blots were then stained using the Protoblot system from Promega.

Immunofluorescence

For indirect immunofluorescence, COS cells were plated on 10 cm tissue culture dishes containing glass coverslips. The cells were transfected with the MDMX or MDMX-S expression plasmids (or the vector control) and 24 h post transfection the plates were washed once in PBS and then fixed with 4% paraformaldehyde in PBS for 20 min followed by an additional rinse in PBS. To permeabilize the cells, the coverslips were treated with PBS plus 0.2% Triton X-100 for 15 min followed by three washes of 5 min in PBS plus 0.2% gelatin. The M2 monoclonal antibody was diluted in PBS plus 0.2% gelatin. A total of 50 μl of diluted antibody was added to a 6 cm culture dish, coverslips containing the fixed and permeabilized cells were placed cell side down on the drop of diluted antibody and incubated for 1.5 h at 37°C. The coverslips were washed three times in PBS plus 0.2% gelatin. Fluorescein conjugated goat anti-mouse IgG (Vector Laboratories) was

diluted to 30 μg/ml in PBS plus 0.2% gelatin. Again 50 μl of diluted antibody were placed in a dish, the coverslips placed cell side down and incubated for 30 min at 37°C. The coverslips were washed in PBS plus 0.2% gelatin (10 min), PBS plus 0.2% gelatin plus 0.05% Tween 20 (10 min) and finally in PBS (10 min). The nuclei were then stained with the DNA dye DAPI. The coverslips were rinsed three times in PBS, once in deionized water, and then they were dried, mounted and analyzed by fluorescence microscopy.

RESULTS

MDMX and MDMX-S Display Differences in Their Subcellular Localization

It has been previously demonstrated that the short isoform of MDMX, termed MDMX-S, has a higher affinity for p53 and a greater ability to block p53-mediated gene expression than MDMX [Rallapalli et al., 1999]. The goal of the studies here was to analyze the activities of MDMX-S in more detail. These activities include its subcellular locale, its ability to regulate gene expression via the DNA damage response pathway mediated by p53 and the identification of domains within MDMX-S that may be responsible for its high affinity interaction with p53. To begin these studies, MDMX and MDMX-S were independently expressed in COS cells and their levels of expression were assessed by immunoblotting of cytosolic and nuclear extracts, using an antibody to the amino terminal Flag epitope. As shown in Figure 1, MDMX is present at higher levels in the cytosolic fractions than in the nuclear fractions. In contrast, MDMX-S is present at slightly higher levels in the nuclear extracts, which is consistent with earlier findings [Rallapalli et al., 1999]. This data indicates that there may be differences in subcellular location of these two proteins. Additionally, it appears from the data that MDMX-S is expressed to a higher level in the cell extracts than MDMX.

Given the recent findings that MDMX is a predominantly cytoplasmic protein [Rallapalli et al., 1999; Jackson and Berberich, 2000; Jackson et al., 2001], it was important to determine if MDMX-S was also cytoplasmic or nuclear. To determine if in fact MDMX-S is located more in the nucleus, immunofluorescence studies were performed on cells ectopically expressing either the tagged MDMX or

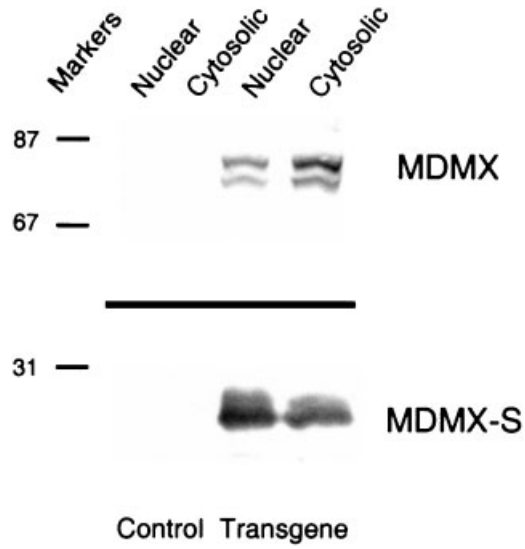

Fig. 1. Ectopic Expression of MDMX and MDMX-S in COS cells. COS cells were transfected with an MDMX expression plasmid (**upper**), an MDMX-S expression plasmid (**lower**) or with the vector alone (**control**). Forty-eight hours post-transfection, the cells were lysed and extracts generated. A total of 50 μg of COS nuclear and cytosolic extract were electrophoresed by SDS-PAGE and blotted onto nylon membrane. Blots were generated and incubated with the M2 antisera specific to the epitope tag, followed by a secondary antibody coupled to alkaline phosphatase. Shown are the developed blots. The bands corresponding to MDMX and MDMX-S are indicated along with the sizes of the markers in kiloDaltons (kDa). The data show that a majority of MDMX-S protein is present in nuclear extracts while a majority of the MDMX protein is present in the cytosolic extracts.

the MDMX-S proteins. As shown in Figure 2A,B, when expressed in COS cells, MDMX-S is predominantly nuclear, and unlike MDM2, appears to be moderately excluded from the nucleoli. In contrast, MDMX is predominantly cytoplasmic as has been described. Figure 2A shows a comparison of MDMX and MDMX-S expression (Fluorescein) along side the DAPI (DNA) stain nuclei. Figure 2B shows a series of cells expressing either MDMX or MDMX-S, where in the side-by side comparison, MDMX-S is primarily nuclear while MDMX is primarily cytoplasmic. The data indicate that MDMX-S and MDMX differ markedly in their subcellular distribution in these cells.

MDMX and MDMX-S Show Differences in Their Ability to Suppress Gene Expression Mediated Directly by p53 and by DNA Damaging Agents

Since the MDMX-S protein is predominantly nuclear when expressed in cells, it would be expected to be more capable of blocking the action of p53. This was found to be the case as

seen in Figure 3A. When NIH3T3 cells are cotransfected with a p53-target promoter and a p53 expression vector, significant transactivation is seen (Fig. 3). However, when these cells are cotransfected with an MDMX-S expression vector along with a p53 expression vector, this transactivation of the promoter is completely blocked. When these cells are transfected with an MDMX expression vector, only partial blockage is evident (Fig. 3A).

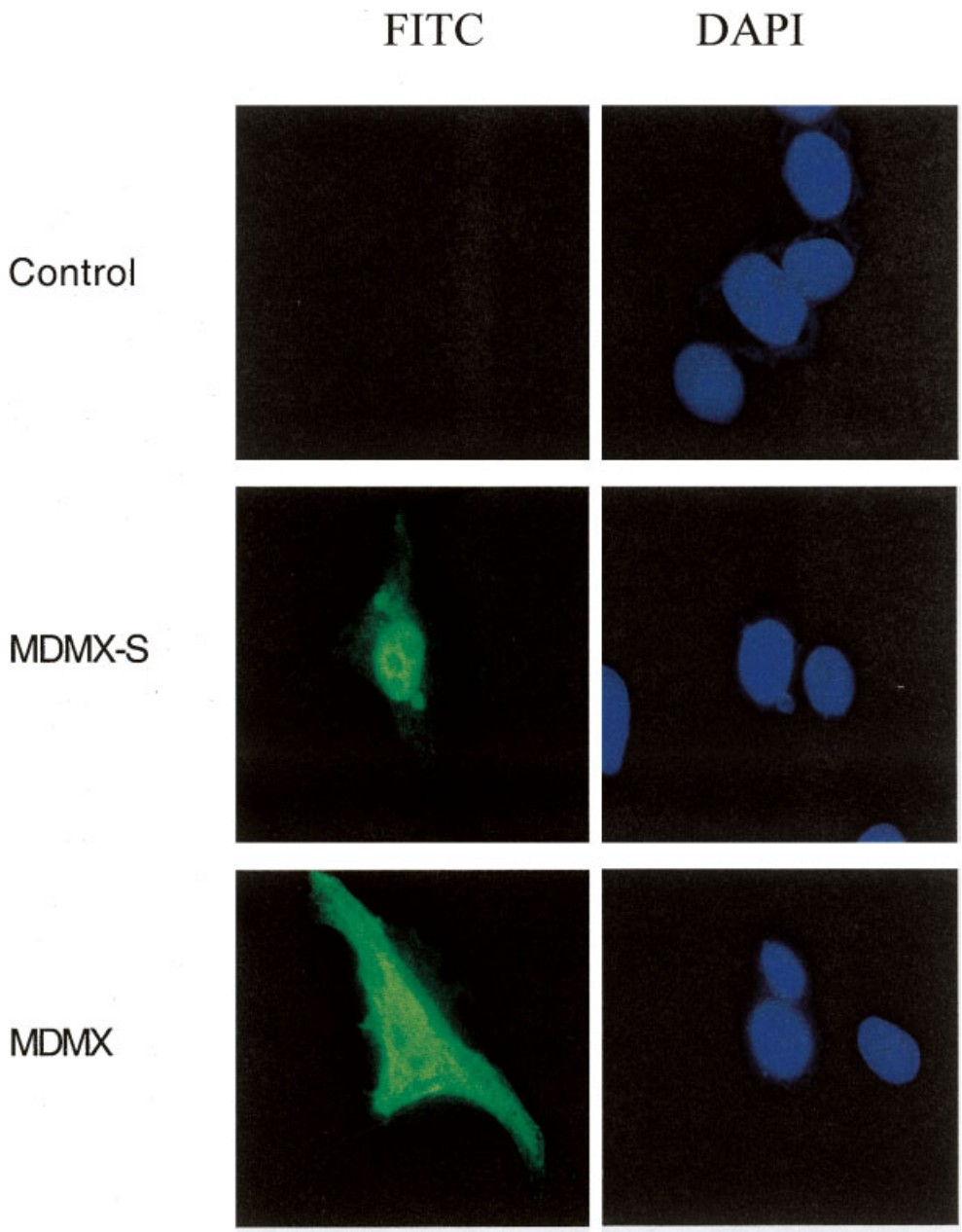

Fig. 2. MDMX-S is predominantly nuclear and MDMX predominantly cytoplasmic when expressed in COS cells. COS cells grown on cover slips were transfected with the vector alone, MDMX or the MDMX-S expression plasmids. Forty-eight hours post-transfection, the cells were fixed and processed for indirect immunofluorescence using an M2 primary antibody directed against the epitope tag and a FITC conjugated secondary antibody. The DNA (nuclei) was then stained with DAPI. **A**: Shown are representative fluorescent positive cells expressing either the vector control, MDMX or MDMX-S following the transient transfection. The data show that MDMX is present primarily in the cytopolasm while the MDMX-S protein is predominantly nuclear but excluded from the nucleoli. **B**: Shown are multiple fluorescent positive cells expressing either MDMX-S (**Left Panels**) or MDMX (**Right Panels**). As in (A) above, the data show that MDMX-S protein is predominantly nuclear while MDMX is present predominantly cytopolasmic. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

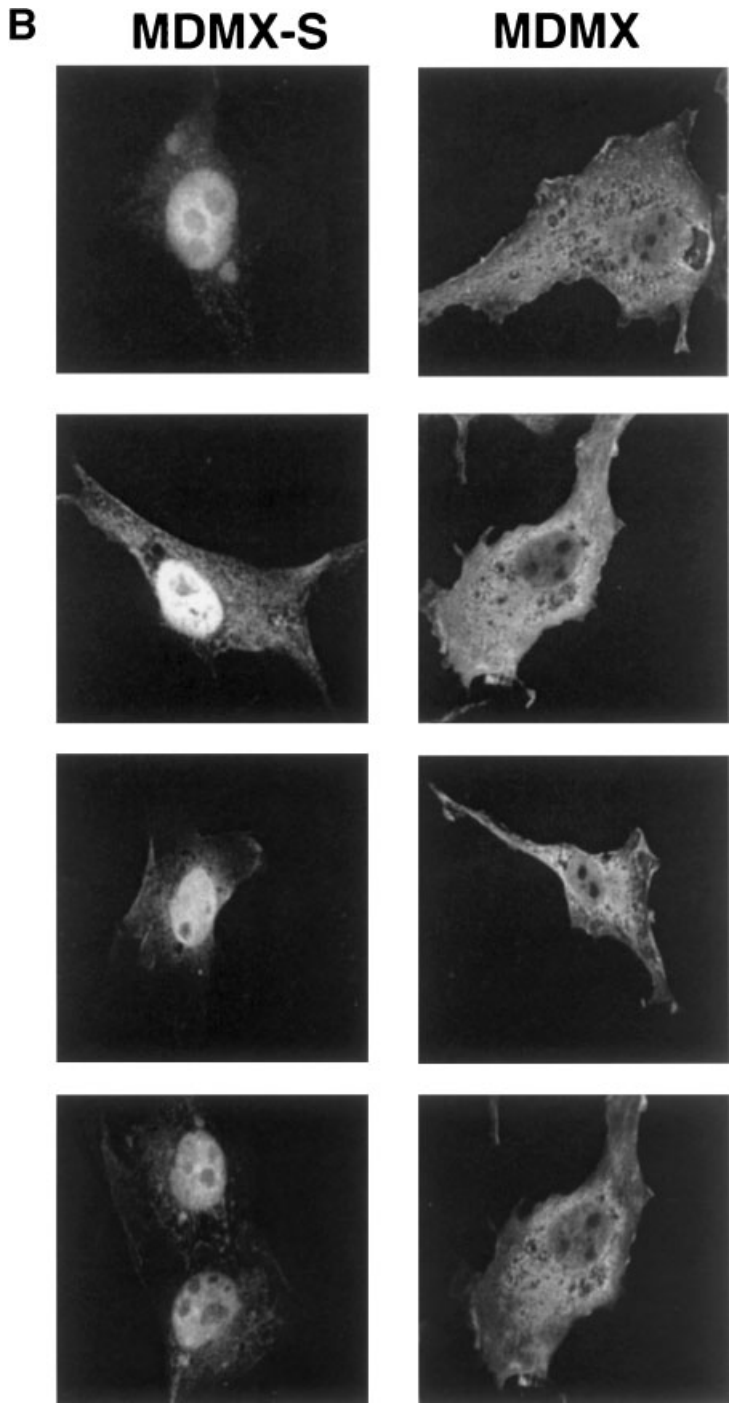

Fig. 2. (*Continued*)

Thus, MDMX-S appears better than MDMX at inhibiting p53-mediated transcriptional transactivation.

Since MDMX-S is better than MDMX at blocking p53 activity, it could be predicted that the induction of a p53-target promoter by DNA damaging agents should be inhibited better by MDMX-S than by MDMX. To perform this experiment, cells were transfected with the p53-target promoter in the presence and absence of an MDMX-S expression plasmid. Additionally where indicated, the cells were treated with 5 μM camptothecin (CPT), a drug well known to induce DNA damage through

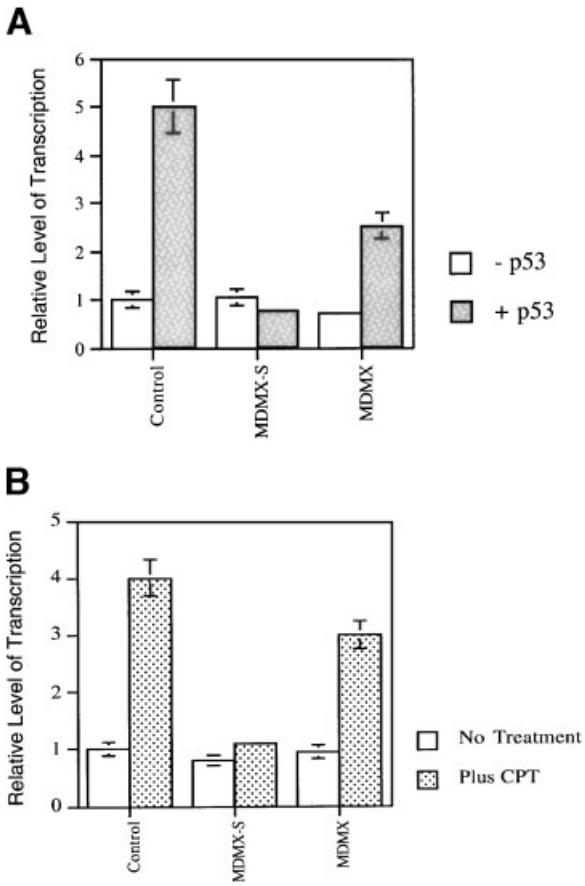

Fig. 3. MDMX-S is a potent suppressor of p53-mediated and DNA damage-inducible transcriptional activation. **A**: NIH3T3 fibroblasts were transfected with the p53-target promoter, pRGCDFOS-lacZ, in the presence or absence of a p53 expression plasmid. Additionally, where indicated, the cells were cotransfected with an MDMX or MDMX-S expression vector. Twenty-four hours post-treatment, the cells were lysed and extracts generated. A total of 10 μg of extract was used in an assay for beta-galactosidase activity. Shown are the relative levels of activity over multiple experiments. The data show that p53 activates transcription from the target promoter, but in the presence of MDMX-S no activation is evident. MDMX is not able to reduce the amount of p53-mediated activation to the level seen by MDMX-S. **B**: NIH3T3 fibroblasts were transfected with the p53-target promoter, pRGCDFOS-lacZ, in the presence or absence of 5 μM camptothecin. Additionally, where indicated, the cells were cotransfected with an MDMX or MDMX-S expression vector. Twenty-four h post-treatment, the cells were lysed and extracts generated. A total of 10 μg of extract was used in an assay for beta-galactosidase activity. Shown are the relative levels of activity over multiple experiments. The data show that the p53 target promoter is activated by treatment of the cells with camptothecin. MDMX-S is able to suppress the activation of the p53 target promoter by camptothecin while MDMX is only partially able to suppress this induction. A plasmid containing the SV40 early promoter linked to luciferase was included in all transfections of the beta-galactosidase constructs (in A and B) so that the beta-gal activity could be normalized for differences in transfection efficiency.

double-stranded DNA breaks during S phase, as a result of its interaction with DNA-topoisomerase I [Logan et al., 1995; Hann et al., 1998]. As shown in Figure 3B, CPT treatment alone was able to induce transcription from the p53-target promoter, indicating that a functional p53 response was active in these cells. As expected, MDM-S expression was able to completely block this activity while MDMX was only able to partially block it (Fig. 3B). Thus, the ability of MDMX-S to inhibit p53-mediated gene expression appears to have a physiological consequence, in that it acts to block the ability of p53 to induce DNA damage responsive genes.

The Novel C-Terminal End of MDMX-S is Required for High Affinity Binding to p53

From the above data, and that published previously [Rallapalli et al., 1999], it is clear that MDMX-S is more efficient at blocking the activity of p53 than MDMX. As demonstrated above, this may at least in part be due to the fact that MDMX-S is targeted more efficiently to the nucleus. An additional effect of MDMX-S on p53 activity is that MDMX-S is better able to bind p53 than full length MDMX. As shown in the graph in Figure 4A (left side), equal amounts of ^{35}S-radiolabelled p53 were applied to small batch columns containing GST, GST-MDMX or GST-MDMX-S. Following extensive washing of the columns and then release of the bound p53 by boiling of the columns in SDS-sample buffer, the amount of bound p53 was determined by gel electrophoresis and phosphorimager analysis of the dried gels. From Figure 4A, it appears that nearly ten-times more p53 binds MDMX-S than MDMX.

To explore the basis for the increased binding affinity of p53 for MDMX-S, we focused on generating carboxy terminal deletions within the MDMX-S protein. While MDMX-S lacks the central and carboxy terminal regions of MDMX it also has a short unique amino acid sequence at its carboxy terminal end (Fig. 4A, right side). It is possible that this unique sequence within MDMX-S plays and important role in p53 binding. To assess the potential function of the short carboxy-terminal peptide, a carboxy terminal deletion mutant was generated in MDMX-S that lacks this portion of the protein. This mutant is shown in Figure 4B (mut no. 1, deletion of 17 amino acid residues). Two other C-terminal mutants were also generated that

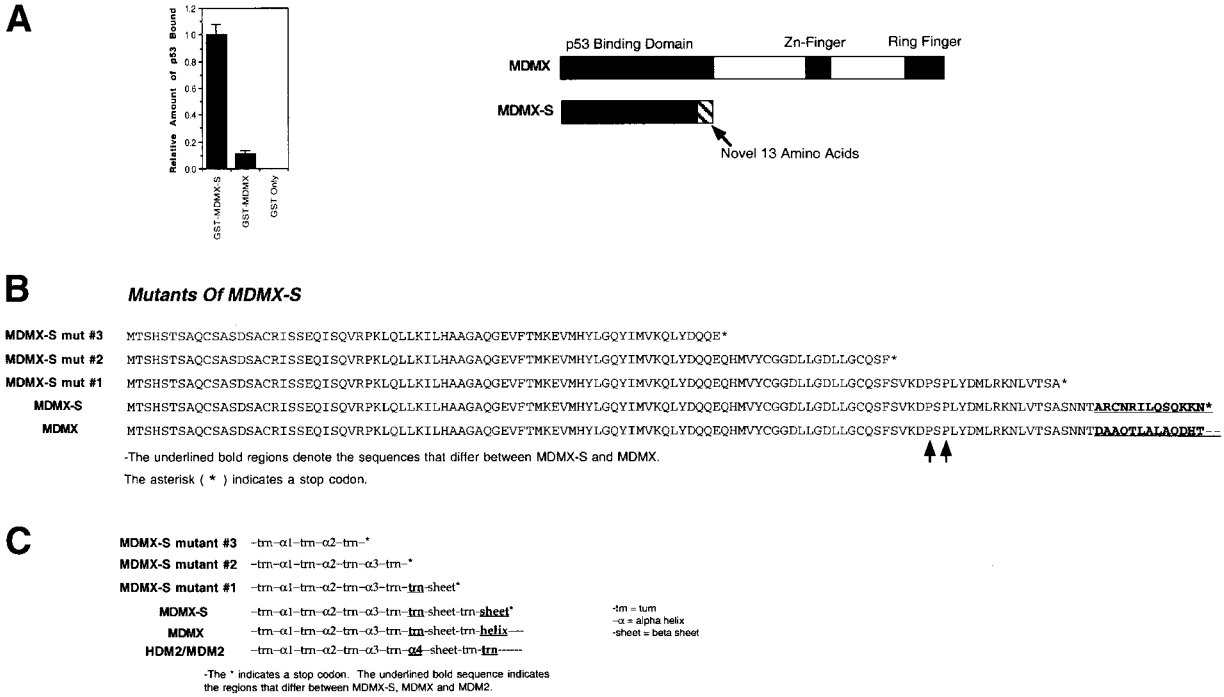

Fig. 4. Wild-type p53 binds to MDMX-S with higher affinity than to MDMX. **A** (**left side**): The p53 protein was radiolabeled by coupled in vitro transcription and translation. The resulting ^{35}S-methionine radiolabeled protein was applied to small batch affinity columns containing GST, GST-MDMX or GST-MDMX-S. The columns were washed extensively in buffer and then boiled directly in SDS-PAGE sample buffer and electrophoresed by SDS-PAGE. The gel was dried and then exposed to X-ray film for autoradiography (the quantitation of the film is shown). The data show that while p53 associated with GST-MDMX-S as apposed to GST-MDMX and that p53 did not associate with GST alone. The amount of GST fusion protein was in 1000-fold excess to p53. **Right side**: The diagram shows an alignment comparison of the MDMX and MDMX-S proteins. **B**: Shown are the amino acid sequences of the three mutants of MDMX-S; mut no. 1, mut no. 2, and mut no.3. The amino acid sequence in bold denotes the sequence that differs between MDMX, and MDMX-S. **C**: The diagram shows the predicted secondary structures of the very amino-terminus of MDMX, MDMX-S and MDM2. The structure for MDM2 was derived from the crystal structure of the amino terminus of the protein [Kussie et al., 1996]. The predicted secondary structures for MDMX and MDMX-S are derived from Robson-Garnier, Chou-Fasman and combined Robson-Garnier/Chou-Fasman algorithms.

lack additional sequence (i.e., mut no. 2 and mut no. 3, Fig. 4B), each deleting 20 amino acids as shown. Figure 4C shows the putative secondary structure of MDMX, MDMX-S, and MDM2, as well as the mutants of MDMX-S. This preliminary structural analysis suggests that MDMX-S differs from both MDMX and MDMX at the carboxy terminal end of the p53-binding domain. The MDMX-S mutants therefore lack the novel sequence at carboxy terminal end of the p53-binding domain.

The mutants were then cloned adjacent to GST, to make GST-fusion proteins, which were expressed in bacteria and purified. To test the affinity of p53 for these mutants, ^{35}S-met radiolabeled p53 (from coupled in vitro transcription/translation) was then applied to small batch columns containing the different GST-MDMX-S fusion proteins mutant forms of MDMX-S (as described in Figure 4A). The result of this binding experiment is shown in Figure 5, where p53 did not detectably bind any of the MDMX-S mutants. The labeled p53 only associated with full-length MDMX-S. These data indicate that the small amino acid residue unique sequence contained within the very carboxy terminus of MDMX-S is needed for optimal association of MDMX-S with p53.

The Novel C-Terminal End of MDMX-S is Required for a High Level of rotein Expression in Cells

From the above it is clear that p53 was bound very poorly if at all to the MDMX-S mutants no. 1, no. 2, and no. 3. It was next important to assess the expression of these mutants in cells, to determine their effects on p53-mediated transcriptional activity. The MDMX-S mutants were epitope tagged (Flag) at their amino termini and cloned into pcDNA3.1, a mam-

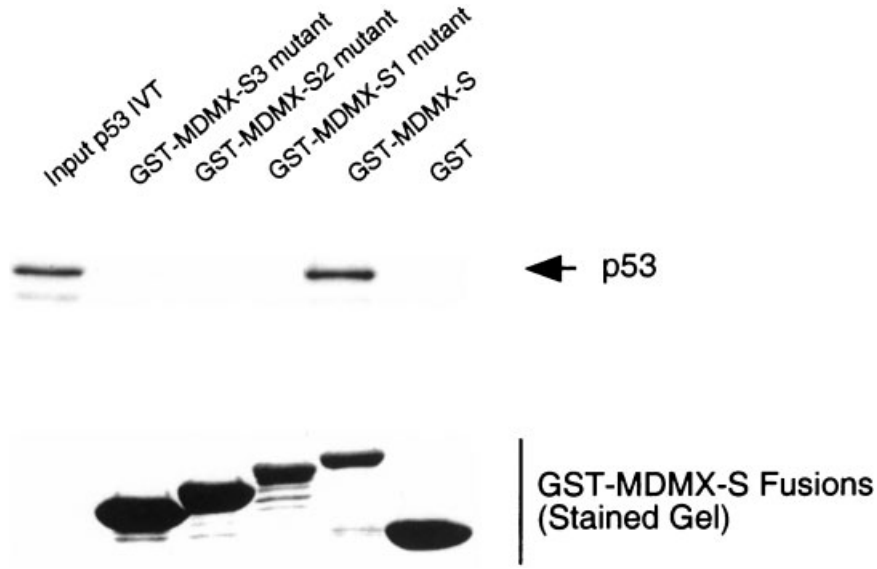

Fig. 5. The novel sequence in MDMX-S is responsible for its high affinity binding to p53. The p53 protein was radiolabeled by coupled in vitro transcription and translation in the presence of ^{35}S-methionine. The resulting radiolabeled protein was applied to small batch affinity columns containing GST, GST-MDMX, GST-MDMX-S, GST-MDMX-Smut1, GST-MDMX-Smut2, and GST-MDMX-Smut3. The columns were washed extensively in buffer and then boiled directly in SDS-PAGE sample buffer and electrophoresed by SDS-PAGE. The gel was dried, exposed to phosphoimager cassette and the amount of p53 bound to the columns was quantitated. The amount of p53 bound to GST-MDMX-S was arbitrarily set at 100%. The data show that only MDMX-S associates with p53. None of the mutants of MDMX-S were able to bind p53.

malian expression vector. These expression constructs were transfected into Phoenix cells (a derivative of 293 cells), extracts were then generated which were assessed by immunoblotting. As seen in Figure 6A, high levels of MDMX-S were detected in the extracts. However, none of the mutants were expressed to high levels and in fact were barely detectable. One possibility for the low level protein expression may be that each mutant is not expressed well at the mRNA level. Therefore RT/PCR analysis was performed on total RNA isolated from the transfected cells. As seen in Figure 6B, nearly equal levels of transcript are detected for MDMX-S and each of the mutants. This data indicates that the lack of protein expression in the transfected cell extracts is not due to lack of mRNA expression.

To determine if the low level of protein expression was in some way due to altered translatability of the mutants, coupled in vitro transcription/translation in the presence of ^{35}S-methionine was performed on each of the mutants, which was then compared to full-length MDMX-S. The resulting proteins were then analyzed by gel electrophoresis, followed by phosphorimage analysis of the dried gels. As seen in Figure 6C, nearly equal levels of each protein are present following in vitro translation, indicating that mutants are likely not defective in translation. In total these data indicate that the very carboxy terminal portion of MDMX-S, which includes the novel 13 amino acids, is needed for high-level protein expression in cells. In the absence of this sequence, mutant MDMX-S proteins are expressed only to low levels.

One possibility for the decreased levels of the mutant forms of MDMX-S is that they are highly susceptible to proteolytic degradation and that perhaps the carboxy terminal domain of MDMX-S made the protein resistant to proteolysis. Cells were therefore transfected with the various MDMX-S mutant expression constructs followed by treatment of the cells with the following protease inhibitors; lactacystin, which inhibits the proteasome [Fenteany and Schreiber, 1998], ALLN, which blocks the proteasome plus calpain and cathespin-like proteases [Rock, 1994] and ALLM, which only blocks calpain and cathepsin-like proteases [Inoue, 1991; Rock, 1994]. Addition of these inhibitors to cells expressing the MDMX-S mutants had no effect on elevating the levels of their cognate gene products, as assessed by immunoblotting (data not shown). These data

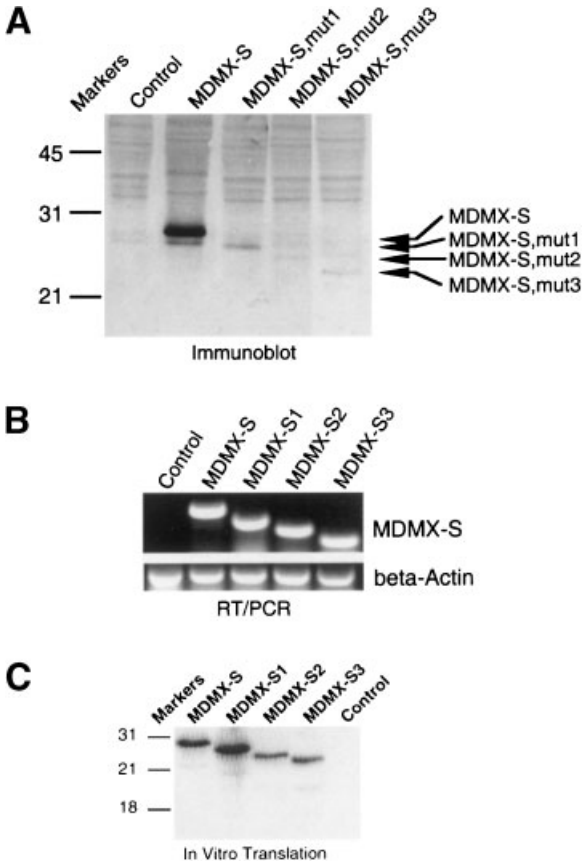

Fig. 6. The carboxy terminal domain of MDMX-S is needed for high-level protein expression in cells. Phoenix cells were transfected with the MDMX-S expression vector or plasmids expressing the mutant MDMX-S proteins (mut no. 1, no. 2, and no. 3 outlined in Figure 4). Twenty-four hours posttransfection, the cells were lysed and either protein extracts were generated or total RNA was isolated. **A**: Shown is an immunoblot of the protein extracts using the FLAG specific monoclonal antibody against the amino terminal epitope tag. The data show that the MDMX-S is present at much higher levels compared to the mutant proteins. **B**: Shown is an RT/PCR analysis using primers specific for MDMX-S and for each mutant. As a control, primers specific for beta-actin were also used in an RT/PCR reaction. The data show that MDMX-S and each mutant are expressed to equal levels. **C**: Using the expression constructs outlined in (A) above, the proteins were radiolabeled by coupled in vitro transcription and translation in the presence of ^{35}S-methionine. The resulting radiolabeled proteins were electrophoresed by SDS-PAGE. The gel was dried and exposed to phosphoimager cassette. The data show that each protein is expressed to equal levels in this in vitro assay.

indicate that these broad groups of proteases do not appear to mediate the reduced protein levels of these mutant cells.

The Carboxy-Terminus of MDMX-S is Required for Optimal Repression of p53 Transcriptional Activity

Since the mutants of MDMX-S do not bind p53 well and are not expressed well in cells, it would be expected that they would not be effective at blocking p53-mediated target gene expression. MDMX-S and mutant expression plasmids were therefore transfected into COS cells in the presence or absence of a p53 expression plasmid plus a p53 target promoter. As seen in Figure 7, p53 is able to induce transcription from this target promoter and coexpression of MDMX-S blocks the ability of p53 to activate transcription. However, the mutants of MDMX-S (mut's no. 1, 2, and 3) are unable to block p53 transcriptional activation. These data are consistent with that shown in Figure 6, in that all the mutant proteins are not expressed well in cells and hence are not able to suppress p53-mediated gene expression.

DISCUSSION

A novel transcript produced by the *MDMX* gene has been previously identified that is nearly identical to the published cDNA sequence, yet has a short internal deletion of 68 base pairs [Rallapalli et al., 1999]. This deletion

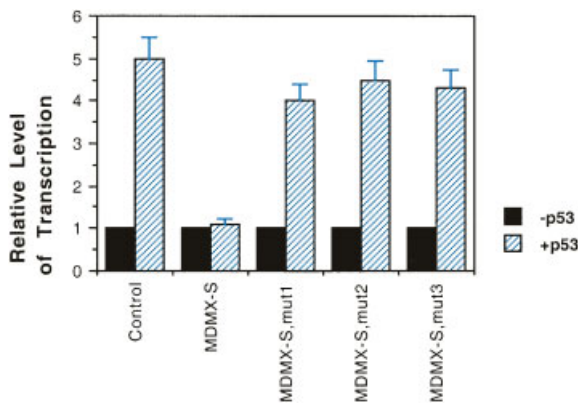

Fig. 7. The carboxy terminal domain of MDMX-S is needed for suppression p53-mediated transcriptional activation in vivo. Phoenix cells were transfected with pRGCDFOS-lacZ vector in the presence or absence of a p53 expression plasmid. Additionally, where indicated, the cells were cotransfected with an MDMX-S expression plasmid or plasmids expressing the mutant MDMX-S proteins (mut no. 1, no. 2, and, no. 3 as in Figure 4). Twenty-four hours posttransfection, the cells were lysed and extracts generated. A total of 10 μg of extract was used in an assay for beta-galactosidase activity. Shown are the relative levels of activity over multiple experiments. The data show that only MDMX-S inhibits p53-mediated transcriptional activation. None of the mutants were able to block p53-induced transactivation. A plasmid containing the SV40 early promoter linked to luciferase was included in all transfections of the beta-galactosidase constructs so that the beta-gal activity could be normalized for differences in transfection efficiency. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

produces a shift in the reading frame after codon 114, resulting in the inclusion of a stop codon at amino acid residue 127 (full length MDMX is 489 residues) [Rallapalli et al., 1999]. This shortened form of MDMX, termed MDMX-S, produces a protein comprised of the p53-binding domain of MDMX with some additional novel amino acid sequence. It appears that MDMX-S expression is high in a majority of mouse and human transformed and immortalized cell lines examined, yet in nontransformed cells and in quiescent cells, MDMX-S transcript levels are very low [Rallapalli et al., 1999]. Additionally, MDMX-S expression in the RNA of normal human tissues is very low (unpublished observations). These data would suggest that expression of MDMX-S may be coordinated with the process of immortalization and/or neoplastic transformation.

Increased expression of MDMX-S in tumor cells correlates well with its properties. MDMX-S is much more effective than either full-length MDMX or MDM2 at inhibiting p53 mediated transcriptional activation and induction of apoptosis [Rallapalli et al., 1999]. This effect is at least partly due to the enhanced ability of MDMX-S to bind p53. MDMX-S binds p53 with higher affinity than MDMX or MDM2. Analysis of the amino acid residues comprising MDMX-S and MDMX indicate interesting differences with respect to an alignment with MDM2. The structure of the very amino terminal 110 amino acids of HDM2 (the human homologue of MDM2) is a 4-helix bundle characterized as a "twisted trough" [Kussie et al., 1996] (the secondary structure of HDM2 is shown in Figure 4C). The activation domain of p53 binds within this trough, and through an "induced-fit" type of interaction thereby forms an alpha helix during the association [Kussie et al., 1996]. Three amino acid residues in p53, F19, W23, and L26, are critical contact points for its association with HDM2 [Kussie et al., 1996; Bottger et al., 1997]. In contrast to HDM2, both MDMX-S and MDMX contain two helix breaking proline residues within the region making up the fourth helix (shown by the vertical arrows in Figure 4B), which implies that a turn exists in this region. This suggests a novel structure for MDMX-S and MDMX with regard to p53 association. Additionally, the novel 13 amino acids of MDMX-S are predicted to form an additional turn followed by a beta sheet, as shown in Figure 4C. These data suggest that MDMX-S has a unique structure, distinct from both MDMX and MDM2.

Consistent with the idea that the very carboxy terminal region of MDMX-S is an essential domain, deletion of the novel amino acids from the very carboxy terminus of MDMX-S leads to a protein that is not able to bind p53 in an in vitro assay. Since this region of MDMX-S may have a unique structure compared to both MDMX and MDM2, it may be that it is this unique structure that is responsible for the high affinity association with p53. However, the nature of the structure of MDMX-S is not known at this point of time.

What is surprising about the studies here is that the very carboxy terminal domain of MDMX-S was also needed for a high level of protein expression in cells. As shown in Figure 5, deletion of the C-terminal domain of MDMX-S significantly reduced the expression level of this protein. This reduction was neither due to a lack of mRNA expression nor due to decreased translatability. While it is possible that the C-terminal domain confers resistance to cellular proteolytic domains, use of specific protease inhibitors targeting the proteasome, calpain and cathepsin-like proteases had no effect on increasing the level of expression of the MDMX-S mutants. At this time the mechanism(s) responsible for the reduction in protein levels in these mutants is not known.

With regard to subcellular location, it was found that MDMX-S was present predominantly in the nucleus of cells, but somewhat excluded from the nucleolus (Fig. 2). This is in contrast to both MDMX and MDM2. MDMX has been shown to reside in the cytoplasm [Rallapalli et al., 1999; Jackson and Berberich, 2000; Jackson et al., 2001] but can be induced to translocate to the nucleus following DNA damage [Li et al., 2002]. MDM2 on the other hand is primarily nucleolar [Li et al., 2002]. While MDMX-S does not contain a strong consensus nuclear localization sequence (NLS), it does contain some potential residues that may be recognized as such (e.g., VRPKL and SQKKN). However, given its small size, it is possible that MDMX-S does not require an NLS, but may simply diffuse into the nucleus, or it may translocate as part of a complex with other proteins.

The central theme of p53 function is that inducers of DNA damage normally result in increases in p53 levels that eventually lead to

cell cycle arrest and/or apoptosis. Expression of MDMX-S could affect the ability of p53 to function optimally in this regard. It is certainly possible that an upregulation of MDMX-S expression in a p53 wild-type cell could potentially make that cell p53 null. It is therefore possible that MDMX-S acts oncogenically, because of its ability to interact with and inactivate p53, acting across a broad spectrum of cell and tissue types. Current efforts are underway to determine if the biological effect of MDMX-S expression is consistent with its role in blocking p53 activity.

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REFERENCES

Balint E, Bates S, Vousden KH. 1999. MDM2 binds p73alpha without targeting degradation. Oncogene 18: 3923–3929.

Boddy MN, Freemont PS. 1994. The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger. TIBS 19:198–199.

Bottger A, Bottger V, Garcia-Echeverria C, Chene P, Hochkeppel HK, Sampson W, Ang K, Howard SF, Picksley SM, Lane DP. 1997. Molecular characterization of the hdm2-p53 interaction. J Mol Biol 269:744–756.

Fenteany G, Schreiber SL. 1998. Lactacystin, proteasome function, and cell fate. J Biol Chem 273(15):8545–8548.

Frebourg T, Barbier N, Kassel J, Ng YS, Romero P, Friend SH. 1992. A functional screen for germ line p53 mutations based on transcriptional activation. Cancer Res 52:6976–6978.

Freedman DA, Wu L, Levine AJ. 1999. Functions of the MDM2 oncoprotein. Cell Mol Life Sci 55:96–107.

Gu J, Kawai H, Nie L, Kitao H, Wiederschain D, Jochemsen AG, Parant J, Lozano G, Yuan Z-M. 2002. Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. J Biol Chem 277:19251–19254.

Hann C, Evans DLC, Fertala J, Benedetti P, Bjornsti MA, Hall DJ. 1998. Increased camptothecin toxicity in mammalian cells expressing Saccharomyces cerevisiae DNA topoisomerase I. J Biol Chem 273:8425–8433.

Haupt Y, Maya R, Kazaz A, Oren M. 1997. MDM2 promotes the rapid degradation of p53. Nature 387:296–299.

Inoue S, et al. 1991. Inhibition of degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in vivo by cysteine protease inhibitors. J Biol Chem 266(20):13311–13317.

Jackson MW, Berberich SJ. 1999. Constitutive MDMX expression during cell growth, differentiation and DNA damage. DNA Cell Biol 18:693–700.

Jackson MW, Berberich SJ. 2000. MDMX protects p53 from MDM2-mediated degradation. Mol Cell Biol 20:1001–1007.

Jackson MW, Lindstrom MS, Berberich SJ. 2001. MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. J Biol Chem 27:25336–25341.

Jordan KL, Haas AR, Logan TJ, Hall DJ. 1994. Detailed analysis of the basic domain of the E2F1 transcription factor indicates that it is unique among bHLH proteins. Oncogene 9:1177–1185.

Kadakia M, slader C, Berberich SJ. 2001. Regulation of p63 function by Mdm2 and MdmX. DNA Cell Biol 20:321–330.

Kubbutat MHG, Jones SN, Vousden KH. 1997. Regulation of p53 stability by MDM2. Nature 387:299–303.

Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavelitch NP. 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science 274:948–953.

Li C, Chen L, Chen J. 2002. DNA damage induces MMDX nuclear translocation by p53-dependent and-independent mechanisms. Mol Cell Biol 22:7562–7571.

Logan TJ, Evans DL, Mercer WE, Bjornsti MA, Hall DJ. 1995. Expression of a deletion mutant of the E2F1 transcription factor in fibroblasts lengthens S phase and increases sensitivity to S phase specific toxins. Cancer Res 55:2883–2891.

Migliorini D, Denchi EL, Danovi D, Jochemsen A, Capillo M, Gobbi A, Helin K, Pelicci PG, Marine J-C. 2002. Mddm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. Mol Cell Biol 22:5527–5538.

Moberg KH, Logan TJ, Tyndall WA, Hall DJ. 1992. Three distinct elements within the murine c-myc promoter are required for transcription. Oncogene 7:411–421.

Mom J, Zambetti GP. 1997. MDM2: "Big brother" of p53. J Cell Biochem 64:343–352.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature 362:857–860.

Ongkeko WM, Wang XQ, Siu WY, Lau AWS, Yamashita K, Harris AL, Cox LS, Poon RYC. 1999. MDM2 and MDMX bind and stabilize the p53-related protein p73. Curr Biol 9:829–832.

Piette J, Neel H, Marechal V. 1997. MDM2: Keeping p53 under control. Oncogene 15:1001–1010.

Rallapalli R, Strachan G, Cho B, Mercer WE, Hall DJ. 1999. A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. J Biol Chem 274:8299–8308.

Rock KL, et al. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78(5): 761–771.

Roth J, Dobbelstein M, Freedman DA, Shenk T, Levine AJ. 1998. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO J 17:554–564.

Sharp DA, Kratowicx SA, Sank MJ, George DL. 1999. Stabilization of the MDM2 oncoprotein by interaction with structurally related MDMX protein. J Biol Chem 274:38189–38196.

Shvarts A, Steegenga WT, Riteco N, Van Laar T, Dekker P, Bazuine M, Van Ham RCA, Van Oordt WVDH, Hateboer G, Van Der Eb AJ, Jochemsen AG. 1996. MDMX: A novel p53-binding proten with some functional properties of MDM2. EMBO J 15:5349–5357.

Shvarts A, Bazuine M, Dekker P, Ramos YFM, Steegenga WT, Merckx G, Van Ham RCA, Van Oordt WVDH, Van Der Eb AJ, Jochemsen AG. 1997. Isolation and identification of the human homolog of a new p53-binding protein, MDMX. Genomics 43:34–42.

Strachan GD, Rallapalli R, Pucci B, Lafond TP, Hall DJ. 2001. A transcriptionally inactive E2F-1 targets the MDM family of proteins for proteolytic degradation. J Biol Chem 276:45677–45685.

Strachan GD, Jordan-Sciutto KL, Rallapalli R, Tuan RS, Hall DJ. 2003. The E2F-1 transcription factor is negatively regulated by its interaction with the MDMX protein. J Cell Biochem 88:557–568.

Thut CJ, Goodrich JA, Tjian R. 1997. Repression of p53-mediated transcription by MDM2: A dual mechanism. Genes Dev 11:1974–1986.