Isolation and Identification of the Human Homolog of a New p53-Binding Protein, Mdmx

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by enhanced degradation of the p53 protein (HPV-E6; Scheffner et al., 1990; Werness et al., 1990), or by as yet unknown ways, possibly involving altered oligomerization of p53 (adenovirus type 12 large E1B, adenovirus type 5 E1A; Steegenga et al., 1995, 1996). Importantly, HPV and HBV have been implicated in human cervical carcinoma and liver carcinoma (Srivastava et al., 1992; Ueda et al., 1995), respectively. In addition, some cellular proteins have been found to bind to p53 and influence its properties as a transcription factor. First, the Wilms' tumor protein WT1, a transcription factor itself, is able to modulate p53-regulated transcription (Maheswaran et al., 1993). Recently, the E2F1 and DP1 proteins were also shown to interact directly with p53 and inhibit its transcription-stimulation function (O'Connor et al., 1995; Martin et al., 1995). Last, p53 was found to complex with the Mdm2 protein (Barak and Oren, 1992; Momand et al., 1992). The association of Mdm2 with p53 completely abrogates all transcription-regulating properties of p53 (Momand et al., 1992; Chen et al., 1995). Mdm2 most likely functions by concealing the transactivation domain of p53 (Oliner et al., 1993). Most interestingly, overexpression of Mdm2 has been found in a variety of tumors, in general correlating with the absence of mutations in the p53 gene (Oliner et al., 1992; Reifenberger et al., 1993; Habuchi et al., 1994; Lianes et al., 1994; Corvi et al., 1995). Overexpression of Mdm2 is apparently sufficient for inhibition of the p53 tumor-suppression function. Thus, inactivation of p53 in human tumors can be achieved by mutation, cytoplasmic retention, or overexpression of Mdm2, or by several viral proteins.

We recently reported the identification of a mouse cDNA encoding a new p53-associating protein that we called Mdmx because of its structural similarity to Mdm2, a well-known p53-binding protein. Here we report the isolation of a cDNA encoding the human homolog of Mdmx. The ORF of the cDNA encodes a protein of 490 amino acids, 90% similar to mouse Mdmx. The homology between Mdmx and Mdm2 is most prominent in the p53-binding domain and the putative metal-binding domains. The Mdmx protein, which, based on SDS–PAGE, has a MW of 80 kDa, can bind p53 in vitro. The human MDMX gene is transcribed in all tissues tested, with high levels in thymus. By fluorescence in situ hybridization analysis we mapped the mouse mdmx gene to chromosome 1 (region F–G) and the human MDMX gene to chromosome 1q32.

INTRODUCTION

The loss of wild-type p53 in human malignancies results in aberrant cell-cycle progression, escape from apoptosis, and enhanced angiogenesis, which all contribute to tumor growth. These biological processes are most likely controlled by p53 through its transcription-regulating function, which, in human tumors, is usually altered due to gene mutations (Holstein et al., 1991, 1994), but can also take place via cytoplasmic retention (Moll et al., 1992, 1995) and by association with other proteins. Tumor antigens of several DNA tumor viruses have been shown to inactivate the function(s) of p53, by a tight association (SV40-LT, adenovirus type 5 large E1B protein, HBV X-antigen; Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow et al., 1982; Zantema et al., 1985; Feitelson et al., 1993),

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responsive gene, mdmx transcription appears not to be regulated in a p53-dependent manner, at least not after UV irradiation. This might suggest that mdmx is not a modifier of p53 after UV irradiation. We now report the isolation of a human cDNA of 2216 bp that encodes the human Mdmx protein of 490 aa. The mouse and human Mdmx proteins have 90% identity at the protein level. We show that the human Mdmx protein binds to p53 in vitro. By FISH analysis we mapped the mouse mdmx gene to chromosome 1 region F–G and its human homolog to chromosome 1q32. We report the identification of a new member of the mdm family that might be involved in human carcinogenesis.

MATERIALS AND METHODS

Northern analysis. A human multitissue Northern blot (Clontech) was hybridized with 32P-labeled probes according to the manufacturer's manual.

Isolation and sequencing of human MDMX cDNA. A cDNA library from the colonic epithelial cell line T84 in a UNI-Zap XR vector (Stratagene) was screened with mouse mdmx cDNA probe according to the protocol supplied by the manufacturer. pBSK plasmids containing the human MDMX cDNA were obtained with R408 helper phage (Stratagene).

Double-stranded DNA was sequenced by a T7 polymerase sequencing kit and AutoRead Sequencing kit (Pharmacia). The sequence reactions were subsequently analyzed manually on polyacrylamide gel and by the ALF (automatic laser fluorescence) DNA sequencing machine (Pharmacia). All the sequence primers were obtained from Isogene Bio-Science B.V.

Isolation of genomic mdmx DNA clones. To obtain genomic mouse mdmx sequences, we screened a genomic mouse library derived from 129/Ola mice in EMBL3A phage using the standard procedure (Sambrook et al., 1989). Subclones were generated by digestion with Sall and by cloning the fragments into Sall-digested pC20R vector. To obtain human MDMX genomic sequences, library N0. 700 (P1 human), ligated into pAd10SacBII (Francis et al., 1994), was screened with a fragment of approximately 560 bp obtained by RT-PCR on mRNA isolated from the human tumor cell line G401 (Weisemann et al., 1987) with a primer set corresponding to nt 460–478/1003–1022. The presence of MDMX-specific sequences in the genomic clones was established by Southern blot analysis with the use of specific cDNA probes (data not shown).

In vitro associations between Mdmx and p53. To obtain in vitro translated Mdmx proteins, the full-length coding regions of mouse and human Mdmx were cloned into pcDNA3 vector (Invitrogen). Human p53 protein was translated in vitro from the modified pET15b/p53 vector (Shvarts et al., 1996) and the human cjun protein from a pBAT vector (Annweiler et al., 1991) containing the human cjun coding region (pBAT-cjun, a gift from Dr. P. Angel, Karlsruhe, Germany).

In vitro transcription/translation was performed with the use of the coupled transcription/translation system obtained from Promega (Madison, WI) in the presence of [35]S-methionine. In vitro translated Mdmx proteins were incubated in the presence or absence of bacterially produced p53 proteins (Shvarts et al., 1996) for 30 min on ice. Subsequent immunoprecipitations were performed as described previously (Steegenga et al., 1995) with a mixture of the monoclonal antibodies PAb122/PAb421 directed against p53, the polyclonal rabbit antisera pAb100 directed against a peptide of mouse Mdmx, or the polyclonal rabbit antisera pAb55 raised against full-length human Mdmx.

To obtain GST-human Mdmx fusion protein, the total coding region of human Mdmx was cloned into vector pRcRc-9, a modified pGEX expression vector (Smith and Johnson, 1988). Approximately 1 µg of GST-Mdmx, bound to beads, was tumbled for 4 h at 4°C with labeled in vitro translated p53. Subsequently the beads were washed three times with binding buffer. Immunoprecipitated and GST-Mdmx-bound proteins were separated on a 9% SDS-polyacrylamide gel, prepared for fluorography with 22.5% PPO (2,5-diphenyloxazole) in dimethylsulfoxide, dried, and exposed to Kodak XAR-5 film at −80°C.

Fluorescence in situ hybridization (FISH). FISH was performed on mouse cells regenerated from a muscle biopsy and on human lymphocytes. Metaphase spreads were prepared via the standard procedures. A total of 400 and 150 ng labeled mdmx probe for mouse and human chromosomes, respectively, and 50x mouse Cot 1 and 50x human Cot 1 DNA (Gibco, Life Technology) was dissolved in 12 µl of a hybridization solution (50% v/v deionized formamide, 10% dextran sulfate, 2x SSC, 1% v/v Tween 20, pH 7.0). Prior to hybridization, the probe was denatured at 80°C for 10 min, chilled on ice, and incubated at 37°C for 30 min allowing preannealing. After denaturation of the slides, probe incubations were carried out under an 20 x 20 mm coverslip in a moist chamber for 45 h. Immunocytochemical detection of the hybridizing probe was achieved with FITC-conjugated sheep-anti-digoxigenin (Boehringer).

For evaluation of the chromosomal spreads a Zeiss epifluorescence microscope equipped with the appropriate filter for visualization of DAPI and FITC fluorescence was used. Digital images were acquired with a high-performance cooled CCD camera (Photometrics, Tucson) and further processed on a computer with the help of the BDS-Image FISH software package (Biological Detection Systems Inc, Rockville, MD).

RESULTS

Molecular Cloning of mdmx cDNA

To identify the human MDMX cDNA we screened a cDNA library from the colon tumorigenic cell line T84.

Construction of human MDMX cDNA

FIG. 1. Schematic representation of human MDMX cDNA clones obtained from a LambdaZap cDNA library from colon carcinoma cell line T84. The Xml1 site in the middle of the cDNA has been used to obtain the human MDMX cDNA containing the complete open reading frame.
FIG. 2. Nucleotide and derived amino acid sequence of human MDMX. (A) Nucleotide sequence of MDMX cDNA and predicted protein sequence. The putative AUG codon is underlined and boldface; putative alternative translation start sites are shown in boldface. Conserved cysteines in the putative zinc-finger and in the RING finger domains together with the core amino acids are given in boldface italics, and a putative nuclear localization signal is underlined. (B) Sequence alignment of Mdm-like proteins.

T84 (Stratagene) with a mouse mdmx cDNA probe (see Materials and Methods). Two independent phage clones, which remained positive after three rounds of purification, were isolated and purified to homogeneity. Sequence analysis showed inserts of 1260 and 2260 bp (clone 1 and clone 2, respectively, in Fig. 1). The shortest construct shows a strong homology with the 5' part of the previously identified mouse mdmx cDNA (Shvarts et al., 1996), including the putative translation start site. Sequencing of the longest construct revealed that in the 3' part of the insert a stretch of 1143 bp shows a strong similarity with the...
part of the mouse mdmx cDNA, including the translation stop codon. Clone 1 and clone 2 showed a 700-bp identical sequence (Fig. 1). Comparing these available sequences with the mouse mdmx cDNA and the human MDM2 cDNA sequence suggested that these 700 bp are a real overlap, allowing us to generate a human MDMX cDNA with the use of an unique like proteins is shown in Fig. 2B. All proteins share the p53-binding site, which is located in their N-terminus. We have no direct evidence that they function as alternative starts in the MDMX mRNA, although in vitro repeat as has been described for the MDM2 cDNA. This human MDMX cDNA contains an open reading frame that encoding a protein of 490 amino acids with strong homology to the mouse Mdmx protein sequence (Fig. 2A). The 3' UTR is similar to that of the human MDM2 cDNA in that it contains an Alu repeat as has been described for the MDM2 cDNA. This human MDMX cDNA contains an open reading frame of 490 amino acids with strong homology to the mouse Mdmx protein sequence (Fig. 2A).

Sequence alignment of human MDMX with human MDM2 revealed, analogous to mouse mdmx, a significant homology at both the DNA and the protein levels. Figure 2A shows the cDNA sequence of human MDMX and the putative open reading frame, encoding a protein of 490 aa. The sequence alignment of the Mdm-like proteins is shown in Fig. 2B. All proteins share the p53-binding site, which is located in their N-terminus.
in pancreas and lung (Ladanyi et al., 1993). A 5.5-kb MDM2 transcript was detected, but we find the MDM2 gene to be almost equally expressed in all tissues on this blot with only a somewhat higher expression in the thymus, but certainly not as prominent as the MDMX transcript.

In Vitro Interaction between Mdmx and p53

We have shown previously that mouse Mdmx can bind p53 in vivo (Shvarts et al., 1996). To investigate the in vitro interaction between human and mouse Mdmx and human p53, we performed two types of experiments. First, we mixed in vitro translated, 35S-labeled Mdmx with bacterially produced and purified p53 protein (see Materials and Methods). After this incubation immunoprecipitations with anti-p53 and anti-Mdmx antibodies were performed.

The results presented in Figs. 4A and 4B demonstrate that in the presence of p53 protein significant amounts of the in vitro translated human and mouse Mdmx proteins are coimmunoprecipitated with a mixture of the anti-p53 antibodies PAb 122/PAb 421. Furthermore, it seems that only the upper band of mouse Mdmx, which is probably generated from the first in-frame AUG, interacts with p53, suggesting that the protein lacking the N-terminal part does not bind p53. Although difficult to see in Fig. 4, the same seems to be true for the human Mdmx.

Expression of MDMX mRNAs in Human Tissues

To determine the expression pattern of human MDMX mRNA we performed Northern blot analysis on a human multi tissue blot. Figure 3A shows that a 10-kb human MDMX transcript is synthesized in all tissues, with the highest amount in the thymus and a very low expression in colon. An additional shorter transcript of approximately 2.2 kb is detectable in testis. We do not know the exact nature of the different mRNAs, but the mRNA expression pattern of the mouse and human is rather conserved and will be discussed in more detail later. Furthermore, expression of human MDMX mRNA was found in all tissues and cell lines tested by RT-PCR (data not shown). We compared the expression of human MDMX with that of MDM2 by reprobing the same blot with an MDM2 probe (Fig. 3B). The human MDM2 gene was previously shown to be expressed in a 5.5-kb transcript, first identified by Oliner et al. (1992) predominantly in skeletal muscle and liver, with somewhat lower levels localization signal of the Mdm2 protein is found at positions 178–183 (RKRHK), in the middle part of the protein, whereas in the human Mdmx protein it is found at positions 442–445 (KRPR) in the C-terminal part.

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IDENTIFICATION OF HUMAN Mdmx

**FIG. 4.** In vitro association between Mdmx and p53. (A, B) Immunoprecipitations on a mixture of in vitro translated, $^{35}$S-labeled Mdmx proteins and bacterially produced p53 with a mouse Mdmx-specific polyclonal antibody pAb100 (αMMDMX), a polyclonal antibody recognizing human Mdmx pAb55 (αHMDMX), or a mixture of PAb 122/421 (αp53); nonimmune mouse antibody (N.I. mouse) was used in control immunoprecipitations as indicated. (C) GST–HMDMX fusion protein or only GST bound to glutathione beads was mixed with either in vitro translated $^{35}$S-labeled p53 protein or cJun protein. After this incubation the beads were washed and bound proteins were separated on SDS–PAGE.

(Szymanska et al., 1996; Forus et al., 1995). The possible amplification of the MDMX gene in these tumors is currently under investigation.

**DISCUSSION**

We report here the isolation and identification of the human counterpart of the mouse Mdmx gene. The human MDMX gene encodes a protein of 490 aa that is 90% homologous to mouse Mdmx (Shvarts et al., 1996). The amino acid sequence predicts a protein with a theoretical molecular weight of 54 kDa. However, on SDS–PAGE human Mdmx was found to migrate as a protein with a molecular weight of approximately 80 kDa. This behavior corresponds to the observation that the Mdm2 protein also runs at a much higher molecular weight on SDS–PAGE than expected from its amino acid sequence. We expect the Mdmx amino acid sequence to be as presented here, since upstream from the putative AUG, in-frame stop codons are found. The apparent molecular weight of human Mdmx is somewhat larger than that earlier found for mouse Mdmx. This might be caused by different posttranslational modifications, e.g., phosphorylation, which is known to occur on the Mdm2 proteins.

A sequence comparison between human MDMX and human MDM2 reveals conservation that is strongest in the p53-binding domain located in the N-terminal part of both proteins. Furthermore, putative functional domains in the C-terminal part of the proteins are highly conserved. These domains consist of a zinc finger and a zinc-binding domain called the RING finger. The latter is thought to be involved in protein–protein interaction (Boddy et al., 1994).
FIG. 5. Chromosomal localization of the mdmx genes by fluorescence in situ hybridization. (A, C) Mapping of the mdmx gene on mouse and human metaphase chromosomes, respectively. Probe DNA, light spots; chromosomes are counterstained with DAPI. (B, D) Giemsa-like banding to identify the chromosome and localize the probe on mouse chromosome 1 F–G and on human chromosome 1q32. At least 20 informative chromosome spreads were used to determine the localization of the mdmx genes.

By Northern blot analysis we detect a 10-kb transcript of human MDMX in most tissues, with a relatively high expression in thymus. Also mouse thymus was found to express relatively high levels of the murine mdmx mRNAs (data not shown). The murine mdmx gene is expressed prominently into two long mRNAs of approx. 10 and 7.5 kb in all tested tissues and a mRNA of 2.2 kb, which is highly expressed in testis and to a very low level in other tissues (Shvarts et al., 1996). An MDMX mRNA of approx. 2.2 kb is also found in human testis. This mRNA is not detected in the other tissues, but if the expression of this mRNA in the other tissues is as low relative to the longer mRNA as in mouse tissues, it might be a detection problem. Thus, the mRNA expression patterns of the human and mouse mdmx genes are rather similar, with the possible exception of the shorter mRNAs in tissues other than testis. It is not firmly established whether the mRNAs detected all contain the coding region here presented. However, we hypothesize that the longer hybridizing RNA band represents not (fully) processed mRNA because of the following reasons. First, the cDNA clone 2 we isolated most likely contains intron sequences, while the library was made from oligo(dT)-primed mRNAs. Second, an entry in the NCBI EST database of 275 bp (L44283), derived from human thymus RNA, is identical over a stretch of 113 bp with the human MDMX cDNA sequence shown here (Fig. 6). From studies on the genomic organization of the mouse gene we know this to be exon 2. Both 5’ and 3’ of these exon sequences the sequence of the EST diverges from the cDNA sequence (Fig. 6), but now shows similarity with intron sequences of the mouse mdmx gene (not shown). These results already suggested that unspliced or not fully spliced mdmx mRNAs are expressed. In addition, we performed an RT-PCR assay on mouse thymus mRNA with one primer derived from exon 2 and another from the following intron. A product of the expected length could be detected, again suggesting that (partly) unspliced mdmx mRNAs are expressed.

The 5’ end of cDNA most likely represents the approximate 5’ end of the mRNA, as indicated by 5’-RACE experiments (Shvarts et al., 1996) and because in the mouse genome just upstream of this region promotor activity was found (R.C.A. van Ham and A.G. Jochemsen, unpublished results). From these results we hypothesize that the long mRNAs represent not fully processed mRNA and that the cDNA sequence we

FIG. 6. Alignment of the 5’-part of the human MDMX cDNA sequence with an EST from the NCBI database (Accession No. L44283).
now show is a representation of the smaller mRNA of 2.2 kb, although other possibilities cannot be excluded. Thus, although we find the human MDMX 10-kb mRNA to be highly expressed in thymus, it remains to be seen whether this has consequences at the protein level. It is not excluded that the unspliced mRNAs also code for Mdmx-like proteins, since in done 2 an in-frame ATG codon is present just eight amino acids in front of the Mdmx-homology region. It will be interesting to find out whether this relatively high mRNA expression will turn out to be important in the differentiation of T-cells or be reflected in a role of MDMX (over-)expression in hematological malignancies, as has been reported for MDM2 (Bueso-Ramos et al., 1993; Maestro et al., 1995; Zhou et al., 1995). MDM2 amplification is found in approximately 30% of human sarcomas (Oliner et al., 1992). A recent analysis of liposarcomas shows 50% amplification in 12q14, most probably involving MDM2. In several liposarcomas (4/14) amplification around 1q32 has also been found, possibly indicating involvement of MDMX (Szynarska et al., 1996). Whether the MDMX gene is present in this amplicon is currently under investigation.

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