The E2F-1 Transcription Factor Is Negatively Regulated by Its Interaction With the MDMX Protein

Gordon D. Strachan,1 Kelly L. Jordan-Sciutto,1 Ravikumar Rallapalli,1 Rocky S. Tuan,2 and David J. Hall2*

1Department of Pathology, University of Pennsylvania School of Dental Medicine, 4010 Locust St, Rm 312 Levy Research, Philadelphia, Pennsylvania 19104-6002
2Cartilage Biology and Orthopaedic Branch, NIAMS, NIH, 9000 Rockville Pike, Bldg., 13, Rm 3W17, MSC 5755, Bethesda, Maryland

Abstract Several proteins with important roles in oncogenesis have been shown to regulate the function of the E2F-1 transcription factor, which is known to activate the expression of genes required for proliferation and apoptosis. Here we identify the MDMX oncoprotein as an E2F-1-binding factor, from a yeast-two hybrid screen using a portion of the E2F-1 protein as “bait.” We demonstrate that the region within MDMX needed for the E2F-1:MDMX interaction is located in the central part of the protein, C-terminal of the p53-binding domain. The region within E2F-1 needed for this association is adjacent to the DNA binding domain. Further, when expressed in vivo or in vitro the MDMX protein migrates as two isoforms on SDS-PAGE, the faster migrating isoform having the stronger affinity for the E2F-1 proteins. It appears that this interaction reduces the ability of E2F-1 to bind DNA. Expression of MDMX along with E2F-1 and Dp-1 in Saos2 cells reduces the ability of E2F-1 to bind to its consensus DNA sequence, without altering E2F-1 protein levels. These data indicate that the MDMX protein is capable of associating with E2F-1 and negatively regulating its DNA binding ability.

J. Cell. Biochem. 88: 557–568, 2003. Published 2003 Wiley-Liss, Inc.

Key words: E2F-1; MDMX; interaction; two-hybrid screen

The E2F-1 factor is among a family of proteins (E2F-1–E2F-6) that regulate the transcription of genes necessary for progression through the late G1- and S-phases of the cell cycle [Martin et al., 1995a, Trimarchi et al., 1998, Muller and Helin, 2000]. Ectopic expression of the E2F-1 protein can result in enhanced proliferation by overcoming the G1/S-phase checkpoint and by driving cells into S-phase. An additional prominent function for E2F-1 is its ability to activate apoptosis when overexpressed [Agah et al., 1997], which is unique to E2F-1 among the E2F family members [DeGregori et al., 1997].

Multiple mechanisms have been identified that are responsible for the activation of apoptosis by E2F-1. For example, E2F-1 is a direct transcriptional activator of the p14ARF, APAF, and p73 genes [Bates et al., 1998, Stiewe and Putzer, 2000]. p14ARF reduces the anti-apoptotic function of MDM2 by targeting MDM2 for degradation in the proteasome [Zhang et al., 1998]. Since MDM2 is a potent blocker of p53 function, this reduction in MDM2 leads to an increase in apoptosis [Haines, 1997, Honda et al., 1997]. The p73 protein on the other hand is a transcription factor that transactivates several known apoptotic gene products, in a similar manner as its homologue-p53 [Arrowsmith, 1999, Kaelin, 1999]. Additionally E2F-1 influences apoptosis by enhancing the degradation of the TRAF2 protein [Phillips et al., 1999], a known inhibitor of apoptosis that functions through caspase signaling [Wang et al., 1998]. Interestingly, all these mechanisms of E2F-1-activated apoptosis require the presence of the DNA binding domain within E2F-1 [Phillips et al., 1997].

Several proteins have been identified that associate with E2F-1 and regulate its function as a transcription factor, which in turn may...
regulate its ability to activate apoptosis. For example, E2F-1 binds to promoters with stronger affinity when it forms a heterodimer with the Dp-1 protein [Bandara et al., 1993]. Heterodimerization of E2F-1:Dp1 occurs through a region that is C-terminal to the E2F-1 DNA binding domain and may include a leucine zipper [Jordan et al., 1994]. Amino terminal to the DNA binding domain is the cyclin A/cdk2 binding domain. The cyclin A/cdk2 kinase binds to E2F-1 and phosphorylates both Dp-1 and E2F-1, resulting in the dissociation of E2F-1 from DNA [Krek et al., 1994, Xu et al., 1994]. Dissociation of E2F-1 from DNA, through the action of cyclin A/cdk2, appears to be a required event for transit through S-phase. For example, expression of mutants of E2F-1 lacking the cyclin A/cdk2 binding domain in cells results in a lengthened S-phase [Krek et al., 1995; Logan et al., 1995, 1996; Stubbs et al., 1999].

In addition to the DNA binding domain and cyclin A/cdk2 binding domains, E2F-1 contains a transactivation domain located at its very C-terminus [Hagemeier et al., 1993]. E2F-1-driven transactivation can be blocked by the retinoblastoma tumor suppressor (pRb), which binds E2F-1 adjacent to the transactivation domain, thereby masking its function [Flemington and Speck, 1993; Helin et al., 1993].

Among the proteins capable of associating with E2F-1 is the MDM2 oncoprotein [Martin et al., 1995b; Loughran and La, 2000]. There is evidence for MDM2 being both an activator and inhibitor of E2F-1 driven transcription. MDM2 has been reported to bind to the E2F-1 C-terminus in vitro and aid in the activation of E2F-1 driven transcription, by preventing the retinoblastoma protein from associating with E2F-1 [Martin et al., 1995b]. However, other reports indicate that the MDM2 protein is an inhibitor of E2F-1-induced apoptosis, through its ability to target both the E2F-1 and Dp-1 proteins for degradation [Loughran and La, 2000].

Since MDM2 associates with E2F-1 it may be expected that any MDM2-related proteins may also interact with E2F-1. In this regard, a protein with strong similarity to MDM2 has been identified that is termed MDMX. Both proteins share some of the same structural and functional characteristics [Shvarts et al., 1996]. For example, each protein has a highly conserved N-terminal p53-binding domain, which mediates repression of apoptosis through the inhibition of the activity of the p53 tumor suppressor [Bottger et al., 1999]. Even though MDM2 and MDMX both repress p53 activity, these proteins have functional differences. The MDM2 protein targets the p53 protein for degradation within the proteasome [Honda et al., 1997], while the MDMX protein stabilizes p53 by repressing MDM2 targeted degradation of p53 [Stad et al., 2000].

While a role for MDMX in p53-mediated functions has been demonstrated, MDMX’s regulation of E2F-1 has not yet been fully elucidated. In this study, we described the isolation of a portion of the MDMX protein in a yeast two-hybrid screen, to identify factors that interact with and regulate the activity of E2F-1. Here we show that the MDMX protein interacts with the E2F-1 protein, near the E2F-1 DNA binding domain, and inhibits E2F-1 from binding DNA.

**MATERIALS AND METHODS**

**Two-Hybrid Yeast Screen**

The two-hybrid yeast screen was performed as previously described [Jordan et al., 1996]. pLexA-E2F-1 (60–121) is expressed from the pBTM116 plasmid containing the coding sequence for amino acids 60–121 of E2F-1 cloned in-frame with the LexA coding sequence. The LexA-E2F-1 (60–121) fusion protein was used as bait to identify E2F-1 binding proteins from a partial length cDNA library, generated from 8.5–10.5-day-old mouse embryos. The cDNA library was cloned adjacent to the activation domain of the herpes virus VP16 transcription factor within the high-copy yeast plasmid-pVP16. The yeast reporter strain L40 (MATa, trp1, leu2, LYS2::lexA-HIS3,URA3::-lexA-lacZ) was grown in rich media YPDA (1% yeast extract, 2% bacto-peptone, 2% glucose, and 0.1 μg/ml adenine) or in synthetic media lacking amino acids for which the yeast cells are prototrophic.

L40 cells were transformed with pLexA-E2F-1 (60–121) by the lithium chloride method. The plasmid was maintained in yeast by selection for the TRP1 marker. The plasmid library DNA was electroporated into yeast and the cells were subsequently plated onto media lacking His (also lacking Ura, Lys, Trp, Leu). Clones that grew on these “His (−)” plates were assayed for beta-galactosidase activity to ensure that the library cDNA did not encode a protein specific...
for the His promoter. The pVP16 plasmid containing the interactive gene was then isolated from the yeast and the unknown cDNA insert was sequenced.

Generation of In Vitro Transcribed/Translated Proteins and GST Fusion Proteins

35S labeled in vitro translated MDMX protein was generated by incubating 1 μg of pRc-CMV-MDMX plasmid along with 35S radiolabeled methionine in a T7 Quick (Promega; Madison, WI) rabbit reticulocyte lysate system for 1 hour at 30°C. The GST-Rb expressing plasmid was a kind gift of Antonio Giordano. GST-Dp-1, GST-E2F-1, GST-E2F-1 (241–437), and GST-E2F-1 (117–437) were created by amplifying the desired cDNAs by PCR then ligating the cDNA in frame behind the GST sequence of the pGEX-2TK plasmid.

GST fusion proteins were generated within the HB101 strain of E. coli. Bacterial cultures transformed with the appropriate pGEX expression plasmid were cultured at 37°C overnight and then diluted 1:10 in selective media and grown for 1 hour at 37°C prior to induction of GST expression with 0.5 mM IPTG. After 3 h of induction, the cells were resuspended in “wash” buffer (10 mM Na2HPO4, pH 7; 30 mM NaCl; 0.25% Tween-20; 10 mM β-mercaptoethanol; 10 mM EDTA; 10 mM EGTA, 2 μg/ml pepstatin; 1 μg/ml leupeptin, and 0.4 mM PMSF) and sonicated. Cell debris was removed by centrifugation and the GST fusion proteins were affinity purified on mini-batch columns (50 μl) of glutathione sepharose beads (Pharmacia) on ice for 30 min. The beads containing the GST fusions were then rinsed three times with 1.0 ml of wash buffer. The GST fusions to be used in the EMSA experiments were dissociated from the sephadex beads by treatment with 5 mM reduced glutathione for 30 min at 4°C.

In Vitro Protein:Protein Association Assay

GST and GST fusion proteins purified and bound to sephadex beads were incubated with equal quantities of 35S radiolabeled in vitro translated proteins in a volume of 0.3 ml of wash buffer for 2 h at 4°C. The GST-fusion sephadex beads were then washed five times with 1.0 ml of wash buffer to eliminate loosely bound proteins and then boiled in SDS sample buffer to dissociate the bound factors. The dissociated proteins were then electrophoresed by SDS–PAGE and were then detected by an autoradiography of the dried gels.

Tissue Culture, Transfections, and the Generation of Extracts

Saos2 and phoenix cells (a derivative of 293 cells) were maintained at 37°C under 4% CO2 and grown in DMEM (BioWhittaker) supplemented with 10% fetal calf serum (Atlanta Biological; Norcross, GA). Cells were split 1:4 the day before transfection. Cells were then transfected by the calcium phosphate method using the Profection kit (Promega) and 20 μg DNA/10 cm plate. Cells were washed twice at 18 h post addition of DNA precipitate and replenished with 10% fetal calf serum.

Cells were harvested 48 h following transfection by scraping in cold PBS. “Cytosolic” extracts were generated by lysing the cells on ice in 0.1% Nonidet P-40, 10 mM Tris (pH 7.9), 10 mM MgCl2, 15 mM NaCl plus the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (1 μg/ml), pepstatin (2 μg/ml), and leupeptin (1 μg/ml). The nuclei were pelleted by centrifugation at 800g for 10 min, and the soluble faction was termed “cytosolic.” The nuclei were resuspended in extraction buffer consisting of 0.42 M NaCl, 20 mM HEPES (pH 7.9), 20% glycerol, phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (1 μg/ml), pepstatin (2 μg/ml), and leupeptin (1 μg/ml) for 10 min on ice followed by centrifugation at 14,000g for 8 min to pellet the residual nuclear material. The supernatant fraction from this centrifugation spin was termed “nuclear” extract.

Immunoblotting and Antibodies

Equal quantities of protein extracts were separated electrophoretically by SDS–PAGE and then transferred onto nitrocellulose blots (Biorad) which were then blocked for 30 min by incubation in 5% dry milk resuspended in TBST buffer [10 mM Tris (pH 8); 150 mM NaCl; 0.05% Tween-20]. The nitrocellulose blots were then incubated in primary antibody for 1 h followed by three washes with TBST for 5 min, then incubated in secondary antibody for 30 min at room temperature. The blots were again washed in TBST three times for 5 min followed by development of the blot, using the Renaissance (NEN Life Sciences; Boston, MA) chemiluminescent kit and X-Omat Blue XB-1 film (NEN Life Sciences).
Primary antibodies for western blot analysis were diluted in 3% dry milk/TBST at the following ratios—1:2,000 for anti-E2F-1 Ab-1 (Calbiochem) and 1:5,000 for M2 anti-FLAG (Kodak). HRP conjugated anti-rabbit IgG (Amersham Life Science) and HRP conjugated anti-mouse Ig (Amersham Life Science) secondary antibodies were diluted 1:10,000 in 3% dry milk/TBST.

**Electrophoretic Mobility Shift Assay (EMSA)**

The probe for the EMSA experiments was created by end labeling two complimentary oligonucleotides, containing a region of the DHFR promoter representing a consensus E2F DNA binding site (5'-GCGAATGCAA TTTCGC- GCCAAACTTGGGGGG-3'), where the underlined sequence represents the E2F binding site [Wade et al., 1995]. The radiolabeled, double-stranded probe was then purified and diluted to a concentration of 20,000 cpm. A double stranded mutant competitor DNA sequence that contains a base change in the E2F binding site, known to impede E2F binding, was included in some binding assays where indicated (5'-GCGAATGCAA TTTC'T'T CGCCAAAC- TTGGGGG-3', where the ''T'' indicates the base change) [Wade et al., 1995].

Equal quantities of either nuclear extract or purified GST fusion protein were incubated with 1 μl of DHFR labeled probe (20,000 cpm), for 20 min at room temperature. Some samples also included either 50 ng of cold double stranded competitor DNA sequence that contains a base change in the E2F binding site, known to impede E2F binding, was included in some binding assays where indicated (5'-GCGAATGCAA TTTC'T'T CGCCAAAC- TTGGGGG-3', where the “T” indicates the base change) [Wade et al., 1995].

RESULTS

**Identification of MDMX as an E2F-1 Associated Factor by a Two-Hybrid Yeast Screen**

The cyclin A/cdk2 kinase is known to associate with the amino terminus of the E2F-1 protein, thereby negatively regulating E2F-1 function. Upon association, this kinase then phosphorylates both the Dp-1 and E2F-1 proteins, resulting in the dissociation of E2F-1 from Dp-1 and from DNA. Through this interaction the amino terminus of E2F-1, therefore plays an important role in determining the rate of progression of cells through S-phase and in the regulation of an S-phase checkpoint [Logan et al., 1994, 1995; Krek et al., 1994, 1995]. Since the N-terminal domain of E2F-1 is a protein–protein interaction domain, we hypothesized that other proteins that regulate E2F-1 function may associate with this region. In this regard, we have previously used the two-hybrid screen to identify two proteins that interact with the very amino terminus of E2F-1 [Jordan et al., 1996].

To further identify proteins that may specifically interact with the E2F-1 amino terminus encompassing the cyclin A/cdk2 binding domain, we performed a yeast-two hybrid screen using amino acids 60–121 of E2F-1, fused to the LexA DNA binding domain as bait [Jordan et al., 1996]. We screened a partial length cDNA library generated from an 8.5–10.5 day mouse embryo, cloned adjacent to the transactivation domain of the VP16 protein from the Herpes virus. An interaction between LexA-E2F-1 (60–121) and the protein produced from the cDNA-VP16 expression plasmid will drive expression of the His-3 and Lac-Z genes that are integrated into the yeast genome (the promoters of His-3 and Lac-Z contain LexA sites).

Yeast containing the LexA-E2F-1 (60–121) expression plasmid was transformed with the cDNA library-VP16 expressing plasmids. First, His-3 expression was assessed by growth on His (-) plates. Colonies were picked, the plasmids containing the unknown cDNAs were isolated and the yeast were retransformed and growth in His (-) media was evaluated. Plasmids were also screened for beta-galactosidase activity, by assaying for blue colony formation in the presence of X-gal (Fig. 1). cDNA’s that grew on His (-) plates and had positive beta-galactosidase activity were sequenced to identify the gene. As seen in Figure 1A, VP16 alone or a beta-catenin-VP16 fusion produced no growth on His (-) plates and were white when cultured on X-gal. However, one cDNA, identified as the MDMX gene (an MDM2 related protein), was able to interact with E2F-1.

The region of MDMX represented by the cDNA pulled out of this two-hybrid yeast screen corresponded to amino acids 129–285 (Fig. 1B) and was expressed in frame with the VP16 transactivation domain. The full-length MDMX cDNA, which encodes the complete MDMX protein, was generated by RT-PCR of poly A+
mRNA from confluent nontransformed NIH3T3 fibroblasts, which also yielded an abundant alternatively spliced transcript encoding a shortened MDMX protein, termed MDMX-S [Rallapalli et al., 1999].

**MDMX Protein Migrates as Two Distinct Bands on SDS–PAGE**

To examine the MDMX:E2F-1 interaction, the MDMX cDNA was cloned into the pCDNA3 mammalian expression plasmid. An epitope tag (FLAG) was cloned just 5' of the MDMX cDNA. We generated radiolabeled MDMX protein by coupled in vitro transcription/translation in rabbit reticulocyte lysates. Electrophoresis of the MDMX protein in a SDS–PAGE revealed that MDMX migrates as two separate bands of 55 and 65 kDa in roughly equal amounts (Fig. 2A). The predicted molecular weight of MDMX is around 54 kDa, suggesting that the larger protein is a post-translationally modified form.

To determine whether the MDMX protein expressed within cells migrated as two bands of comparable sizes to the in vitro translated protein, phoenix cells (a 293 derivative) were transiently transfected with the MDMX expression plasmid. Extracts from these cells were generated, separated by SDS–PAGE and transferred to nitrocellulose (Fig. 2A). The gel was transferred to nitrocellulose and the MDMX proteins from the transfected extracts were detected by incubation of the blot using the anti-FLAG M2 primary antibody specific for the 5' epitope of MDMX. The bands were detected by chemiluminescence (short exposure). The radiolabeled in vitro translated MDMX was detected by placing the blot on film (long exposure). Alignment of the exposed films is shown. B: Saos2 cells were transfected with either MDMX expression plasmid or a control plasmid (pcDNA3). “Cytosolic” and “Nuclear” extracts were generated and analyzed (25 μg/lane) for MDMX protein by immunoblot analysis using the anti-FLAG M2 primary antibody (Kodak) specific for the 5' epitope of MDMX. The blots were then processed for chemiluminescence. The MDMX bands are highlighted by the two arrows.
containing cell extracts expressing ectopic MDMX (Fig. 2A). Exposure of the blot to film and alignment of the bands indicated that the two forms of MDMX produced by in vitro translation migrate to the same positions as that produced by expression in vivo and were therefore identical size to the in vitro translated MDMX proteins. Additionally, since we were able to detect both MDMX forms using an antibody specific for the 5’ FLAG epitope, and since the epitope is immediately adjacent to the ATG, the faster migrating MDMX protein is not the result of translation initiation from an internal ATG.

The MDMX protein was also transiently expressed within Saos2 cells to determine if there was any cell type specificity of expression of the two MDMX isoforms. While “cytoplasmic” extracts generated from transfected Saos2 cells contained the slower migrating MDMX form, the “nuclear” extracts contained nearly equal quantities of the two migrating MDMX proteins (Fig. 2B). The difference in levels of the two MDMX protein forms between the nuclear and cytosolic extracts suggests that there may be a difference in subcellular localization or solubility of the MDMX proteins.

**MDMX Independently Interacts With Both The \( \gamma E2F-1 \) and pRb Proteins**

Since only a portion of the MDMX protein was pulled out of a two-hybrid yeast screen, we wanted to determine whether a full-length MDMX protein could interact with a full-length E2F-1 protein in an in vitro binding assay. We therefore incubated an equal quantity of \( ^{35} \)S-radiolabeled in vitro translated MDMX protein with either GST or a GST-E2F-1 fusion protein bound to glutathione-sephadex beads. The beads were then extensively washed and the remaining bound protein was eluted by boiling the beads in SDS sample buffer followed by electrophoresis of the eluate on a SDS–PAGE. The amount of bound MDMX was determined by exposure of the dried gel to X-ray film, as shown in Figure 3. A significant proportion of the MDMX protein remained bound to GST-E2F-1, yet MDMX did not bind to the GST control. Interestingly, the faster migrating MDMX protein bound to GST-E2F-1 with an apparent higher affinity than the slower migrating MDMX form.

The MDMX homologue, MDM2, has previously been shown to interact with the carboxy terminus of E2F-1 as well as with the pRb and Dp-1 proteins [Martin et al., 1995a; Xiao et al., 1995]. Since MDMX and E2F-1 were able to interact in an in vitro binding assay, we wanted to determine whether MDMX could independently associate with Dp-1 and pRb in the same assay. As shown in Figure 3, MDMX appears to bind with a higher affinity to pRb than to E2F-1. Again, the faster migrating MDMX protein bound to pRb more efficiently than the slower migrating form, as was the case with E2F-1. The data also show that the MDMX protein did not associate well with the Dp-1 protein. Thus, MDMX is able to interact with both E2F-1 and the pRb tumor suppressor protein.

It has been previously shown that the MDM2 protein interacts via its N-terminal p53-binding domain with E2F-1 [Martin et al., 1995b], which is homologous both structurally and functionally to the N-terminus of MDMX [Bottger
et al., 1999]. To further characterize the E2F-1:MDMX interaction, we created a MDMX mutant protein, termed MDMXd114, which does not contain the conserved p53-binding domain, but should contain the E2F-1 interaction region (Fig. 4A). Additionally, we also used the MDMX-S protein in this same assay. MDMX-S is an alternatively spliced form of MDMX that contains only the N-terminal p53-binding domain (Fig. 4A) [Rallapalli et al., 1999]. Unlike the full-length MDMX protein, the in vitro translated MDMX-S and MDMXd114 proteins migrated primarily as single bands on SDS–PAGE (Fig. 4B). This suggests that any modification to MDMX is dependent upon either a complete protein (i.e., it may require both amino and carboxy termini) or that a domain essential for producing the two bands is disrupted in both mutants (i.e., is located near amino acid residue 114).

To determine whether the conserved p53-binding domain of MDMX or the C-terminus of MDMX plays a role in the interaction with E2F-1, we assessed the affinities of MDMXd114 and MDMX-S for E2F-1 in an in vitro binding assay. A much greater proportion of MDMXd114 bound to GST-E2F-1 than to GST alone while only a slightly greater proportion of MDMX-S remained bound to GST-E2F-1 compared to the GST control (Fig. 4C). This suggests that the part of MDMX involved in the MDMX:E2F-1 interaction lies predominantly outside the N-terminal p53-binding domain, which is consistent with the results of the two-hybrid yeast screen. By performing the same type of in vitro binding assay described above, we found that the MDMXd114 mutant also retained its ability to associate with pRb, as shown in Figure 5.

It was next important to perform the converse of the above experiments; to determine whether MDMX could interact with domains within the E2F-1 protein. We performed similar in vitro binding assays as just described, between radiolabeled full-length MDMX protein and GST fusion proteins containing either amino acids 117–241 or amino acids 241–437 of E2F-1 (Fig. 6A,B). The MDMX protein was capable of interacting with amino acids 117–241 of E2F-1, but not amino acids 241–437. Interestingly,
amino acids 117–241 of E2F-1 span the DNA binding domain. Therefore, at least part of the MDMX:E2F-1 interaction appears coincident with, or at least in close proximity to, the E2F-1 DNA binding domain.

**MDMX Inhibits E2F-1 DNA Binding**

Since MDMX associates with the E2F-1 protein near its DNA binding domain, it is likely that the MDMX protein could affect the affinity of E2F-1 protein for DNA. We therefore mixed in vitro translated MDMX protein with the GST-E2F-1 fusion protein and then performed gel shift assays to assess any affects of MDMX on E2F-1 DNA binding. As seen in Figure 7A, purified GST-E2F-1 is capable of binding a 32P-radiolabeled DHFR probe containing a consensus E2F binding site; purified GST protein alone is not able to bind the probe. Incubation of an E2F-1 specific antibody with the binding reaction resulted in a supershift of the probe (Fig. 7A), demonstrating that the protein:DNA complex contains the E2F-1 protein, whereas addition of a control antibody (against SV40 T antigen) had no effect on the protein:DNA complex. To test whether MDMX could affect E2F binding to DNA, additional gel shift assays were performed. The addition of in vitro translated MDMX protein to the binding reaction greatly reduces the ability of GST-E2F-1 to shift probe, as shown in Figure 7B, suggesting that the MDMX protein is capable of inhibiting the E2F-1:DNA interaction. As a control, reticulocyte lysate alone did not affect the GST-E2F-1:DNA interaction (Fig. 7B). These data indicate
that MDMX has an inhibitory effect on the binding of E2F-1 to DNA in vitro.

Since MDMX could inhibit the GST-E2F-1 protein from binding to DNA in an in vitro mixing experiment, we wanted to determine whether MDMX had the same effect on the DNA binding ability of E2F-1 when both are expressed in cells. We therefore transiently expressed the MDMX, E2F-1, and Dp-1 proteins in Saos2 cells (Dp1 was coexpressed to get optimal binding in vivo). The cells were harvested, and “nuclear” extracts were generated which were used in gel-shift assays with the DHFR probe. Saos2 cells that were transfected with an E2F-1 expression plasmid alone did not show any increase in the amount of binding to probe compared to a control pCDNA3 plasmid (Fig. 8A). However, the coexpression of E2F-1 with its heterodimer partner Dp-1 enhanced the amount of binding to the probe (Fig. 8A). Importantly, the expression of MDMX along with E2F-1 and Dp-1 reduced the amount of protein:DNA complex formed, to levels comparable to that of the pCDNA3 control extracts. Coexpression of MDMX with E2F-1 alone did not alter the level of bound probe from that of E2F-1 alone. This data indicates the MDMX inhibits the binding of E2F-1 to DNA in vivo.

Next we wanted to determine whether the decrease in binding to the DHFR probe, by MDMX protein coexpression, was the result of a specific loss of E2F-1 binding to the probe. We first performed a supershift assay using an E2F-1 specific antibody added to the binding reaction, to demonstrate that the increase in protein binding to the DHFR probe by the coexpression of E2F-1 and Dp-1 was due to an increase in formation of an E2F-1:DNA complex.

Fig. 8. MDMX expression inhibits the DNA binding ability of E2F-1 in Saos2 cell extracts. A: Equal quantities of “nuclear” extracts (5 µg) generated from transfected saos2 cells were incubated with DHFR probe in a gel shift experiment. Transfections contained combinations of E2F-1 (5 µg), Dp-1 (5 µg), MDMX (15 µg) expression plasmids and enough control pCDNA3 to bring the amount of DNA/sample to 25 µg. The arrow indicates the position of the E2F-1:DNA complex. B: Equal quantities of nuclear extracts (5 µg) from control transfected (pCDNA3) and E2F-1 + Dp-1 transfected saos2 cells were incubated in a gel shift experiment with and without an anti-E2F-1 antibody (1 µl, Santa Cruz). The arrow indicates the position of the supershifted E2F-1:DNA complex. C: Equal quantities of “nuclear” extracts generated from transfected saos2 cells (5 µg) were incubated with DHFR probe and 50 ng of either wildtype or mutant unlabeled DHFR competitor DNA. Transfections contained the expression plasmids that are indicated above each lane, the total amount of DNA/transfection was 25 µg. The arrow indicates the position of the E2F specific complex.
The addition of an E2F-1 specific antibody to the binding reaction caused a supershift of the probe in the extracts expressing E2F-1 and Dp-1, compared to control Saos2 extracts (Fig. 8B), demonstrating that the increase in binding is due to an increase in formation of an E2F-1:DNA complex.

As an additional test, competitor DNA’s were added to the binding reactions. When a wild-type competitor (Fig. 8C, top) was added to the reactions, binding was significantly reduced in the control, the E2F1/Dp1 and the E2F1/Dp1/MDMX transfected extracts (Fig. 8C, bottom). In contrast, addition of a mutant competitor (Fig. 8C, top) did not affect the relative levels of E2F binding. In the presence of the mutant competitor, the E2F1/Dp1 extracts showed significantly higher protein binding to DNA than the E2F1/Dp1/MDMX extracts (Fig. 8C, bottom). These data again demonstrate that the increase in binding is due to an increase in formation of an E2F-1:DNA complex.

**MDMX Expression Does Not Affect the Steady-State Cellular Levels of E2F-1 Protein**

It has previously been shown that the MDMX homolog, MDM2, is capable of targeting the E2F-1 protein for degradation in Saos2 cells [Loughran and La, 2000]. In order to determine whether the MDMX-mediated reduction of E2F-1 DNA binding was the result of a reduction in E2F-1 protein levels, we performed a western blot analysis of Saos2 “nuclear” and “cytoplasmic” extracts to measure the relative E2F-1 protein levels (Fig. 9A). Unlike what has been reported for MDM2, coexpression of MDMX with E2F-1 does not reduce the amount of E2F-1 protein in the Saos2 cell line. In fact if anything, it appears that MDMX may slightly increase the steady-state level of E2F-1. Therefore, the reduction in E2F-1 binding to the DHFR probe is likely not due to a reduction in E2F-1 protein levels. In contrast to the above findings, the expression of E2F-1 did reduce the amount of steady-state levels of the MDMX protein, as shown in the immunoblot in Figure 9B. This indicates that the E2F-1 protein targets MDMX for degradation as has been recently shown [Strachan et al., 2001].

**DISCUSSION**

Here, we have isolated a portion of the MDMX gene product in a screen for proteins that associate with the E2F-1 transcription factor. We have shown that the full-length MDMX and E2F-1 proteins are able to interact with each other in an in vitro binding assay. Partial mapping of the MDMX-binding domain within E2F-1 revealed an importance for the interior portion of E2F-1, in close proximity to the E2F-1 DNA binding domain. By an in vitro assay, residues 117–241 of E2F-1 were identified as being required for the association. No affinity of MDMX for the C-terminus of E2F-1 (i.e., amino acid residues 241–437) was detected. This is in contrast to MDM2, an MDMX homologue, which has previously been shown to interact with the pocket protein-binding domain near
the C-terminus of E2F-1. The close proximity of the MDMX interaction domain to the DNA binding domain within E2F-1 suggests that the interaction between the MDMX:E2F-1 proteins may explain the ability of MDMX to inhibit the ability of E2F-1 to bind DNA, by both an in vitro and in vivo assay. It is possible that MDMX sterically hinders the interaction of E2F-1 with DNA.

While E2F-1 associates with the conserved p53-binding domain on MDM2 [Martin et al., 1995b], we found that the region of MDMX with highest affinity for E2F-1 is C-terminal to this p53-binding domain. In this regard, MDMX-S, an MDMX isoform that contains only the conserved p53-binding domain, does not associate well with E2F-1. Yet MDMXd114, which spans residues 114–489, interacts moderately well with E2F-1. These data suggest that the interior part of MDMX is needed for optimal association with E2F-1. Since the regions within MDMX and MDM2 that are needed for association with E2F-1 are distinct, it is likely that MDMX and MDM2 have different effects on E2F-1 function, which appears to be the case.

In this regard, we find that the MDMX protein is capable of inhibiting the binding of the E2F-1 protein to DNA. The results of in vitro mixing experiment indicate that inhibition of E2F-1 DNA binding is a direct effect of MDMX and not the result of an indirect biological event such as a change in gene expression or proliferation for example. We also demonstrated that coexpression of MDMX with E2F-1 in Saos2 cells is sufficient to reduce the DNA binding ability of E2F-1, demonstrating that this characteristic of MDMX is retained in an in vivo setting.

In terms of the biochemistry of MDMX, the protein migrates as two distinct bands on a SDS–PAGE. Interestingly, it was found that the faster migrating form has higher affinity for both the E2F-1 and pRb proteins. The two forms of MDMX were observed when it was expressed in cells and when it was generated by coupled in vitro transcription/translation. While we were unable to identify the cause of the two forms, we were able to rule out a conserved alternate translation initiation site, since both forms were detectable by immunoblot, using an antibody specific for the amino-terminal FLAG epitope tag. Further, acid phosphatase treatment of the extracts did not alter the migration pattern of these two MDMX forms, suggesting that they are not due to phosphorylation (data not shown). Additional work will be required to determine the nature of the modifications that result in the generation of these two forms of MDMX and to answer the question as to why one form of MDMX has a higher affinity for E2F-1 (and pRb), since it is likely that it is this form that exerts the negative effect on E2F-1 function.

An additional characteristic of MDMX that makes it distinct from that of MDM2 is with regard to its targeted degradation of E2F-1. It is known for example that MDM2 is capable of decreasing E2F-1 protein levels in Saos2 cells in the presence of Dp-1 protein [Loughran and LaThangue, 2000]. This is likely due to the fact that the MDM2 protein has been shown to be an E3 ubiquitin ligase that specifically targets the destruction of the p53 tumor suppressor. This function is not conserved in the MDMX protein, in fact MDMX may block the E3 ubiquitin ligase function of MDM2 [Sharp et al., 1999]. In this regard, we show here that coexpression of E2F-1 and MDMX did not lead to a drop in E2F-1 protein levels. In fact, the converse was true, E2F-1 appears to target MDMX for breakdown [Fig. 9 and Strachan et al., 2001]. From the data presented here it appears that E2F-1 and MDMX negatively affect each other. MDMX inhibits the ability of E2F-1 to bind DNA and E2F-1 targets MDMX for degradation.

ACKNOWLEDGMENTS

We thank Dr. Garry Nolan for kindly providing the Phoenix cells, Antonio Giordano for providing pCMV-Rb and Kristian Helin for providing the Dp-1 expression plasmid. G.S. was supported by the Foerderer Foundation.

REFERENCES

Agah R, Kirshenbaum LA, Abdellatif M, Truong LD, Chakraborty S, Michael LH, Schneider MD. 1997. Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo. J Clin Invest 100(11):2722–2728.

Arrowsmith CH. 1999. Structure and function in the p53 family. Cell Death Differ 6(12):1169–1173.

Bandara LR, Buck VM, Zamanian M, Johnston LH, La NB. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J 12(11):4317–4324.

Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, Vousden KH. 1998. p14ARF links the tumour suppressors RB and p53. Nature 395(6698):124–125.

Bottger V, Bottger A, Garcia C, Echeverria P, Chene HK, Hochkeppel W, Sampson K, Ang SF, Howard SM,
Picksley DP. 1999. Comparative study of the p53-mdm2 and p53-MDMX interfaces. Oncogene 18(1):189–199.

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci USA 94(14):7245–7250.

Flemington EK, Speck SH. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 90(15):6914–6918.

Hagemeier C, Cook A, Kouzarides T. 1993. The retinoblastoma protein binds E2F residues required for activation in vivo and TBP binding in vitro. Nucleic Acids Res 21(22):4998–5004.

Haines DS. 1997. The mdm2 proto-oncogene. Leuk Lymphoma 26:227–238.

Helin K, Harlow E, Futtaey A. 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 13(10):6501–6508.

Honda R, Tanaka H, Yasuda H. 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 420(1):25–27.

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(4):1177–1185.

Jordan KL, Evans DL, Mercer WE, Bjornsti MA, Hall DJ. 1996. Isolation of two novel cDNAs whose products associate with the amino terminus of the E2F1 transcription factor. Biochemistry 35(38):12320–12328.

Kaelin WG, Jr. 1999. The p53 gene family. Oncogene 18(53):7701–7705.

Krek W, Xu G, Livingston DM. 1995. Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83(7):1149–1158.

Logan TJ, Jordan KL, Hall DJ. 1994. Altered shape and cell cycle characteristics of fibroblasts expressing the E2F1 transcription factor. Mol Biol Cell 5:667–678.

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

Logan TJ, Jordan KL, Hall DJ. 1996. Constitutive expression of the E2F1 transcription factor in fibroblasts alters G0 and S phase transit following serum stimulation. Biochem Cell Biol 74:21–28.

Loughran O, LaTanghie NB. 2000. Apoptotic and growth-promoting activity of E2F2 modulated by MDM2. Mol Cell Biol 20(6):2186–2197.

Martin K, Trouche D, Hagemeier C, Kouzarides T. 1995a. Regulation of transcription by E2F1/DP1. J Cell Sci Suppl 19:91–94.

Martin K, Trouche D, Hagemeier C, Sorensen TS, La NB, Thangue T. 1995b. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. Nature 375(6533):691–694.

Muller H, Helin K. 2000. The E2F transcription factors: Key regulators of cell proliferation. Biochim Biophys Acta 1470(1):M1–M12.

Phillips AC, Bates SKM, Ryan K, Helin KH. 1997. Induction of DNA synthesis and apoptosis are separable functions of E2F-1. Genes Dev 11(14):1853–1863.

Phillips AC, Ernst MK, Bates S, Rice NR, Voussen KH. 1999. E2F1 potentiates cell death by blocking antiapoptotic signaling pathways. Mol Cell 4(5):771–781.

Rallapalli R, Strachan G, Cho B, Mercer EW, 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(12):8299–8308.

Sharp DA, Kratowicz 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 T, Laar P, Dekker M, Bazeunie RC, van W, Ham G, van AJ, der AG. 1996. MDMX: A novel p53-binding protein with some functional properties of MDM2. EMBO J 15(19):5349–5357.

Stad R, Ramos YF, Little N, Grivell S, Attema J, van AJ, der A. 2000. Hdmx stabilizes Mdm2 and p53. J Biol Chem 275(36):28029–28044.

Stiewe T, Putzer BM. 2000. Role of the p53-homologue p73 in E2F1-induced apoptosis. Nat Genet 26(4):464–469.

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.

Stubs MC, Strachan GD, Hall DJ. 1999. An early S phase checkpoint is regulated by the E2F1 transcription factor. Biochem Biophys Res Commun 258:77–80.

Trimarchi J, Fairchild MB, Verona R, Moberg K, Andon N, Lees JA. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. Proc Natl Acad Sci USA 95(6):2850–2855.

Wade M, Blake MC, Jambou RC, Helin K, Harlow E, Azizkhan JC. 1995. An inverted repeat motif stabilizes binding of E2F and enhances transcription of the dihydrofolate reductase gene. J Biol Chem 270(17):9783–9791.

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. 1998. NF-kappaB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281(5357):829–835.

Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR, Livingston DM. 1995. Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature 375(6533):694–698.

Xu M, Sheppard KA, Peng CY, Yee AS, Piwnica H. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol 14(12):8420–8431.

Zhang Y, Xiong Y, Yarbrough WG. 1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 92(6):725–734.