A Transcriptionally Inactive E2F-1 Targets the MDM Family of Proteins for Proteolytic Degradation*

Gordon D. Strachan, Ravikumar Rallapali, Bruna Pucci, Toulouse P. Lafond, and David J. Hall§

From the Departments of Orthopedic Surgery and Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

E2F-1-activated transcription promotes cell cycle progression and apoptosis. These functions are regulated by several factors including the E2F-1-binding protein MDM2 and the retinoblastoma protein pRb. Using a yeast two-hybrid screen we have identified the MDM2-related protein, MDMX, as an E2F-1-binding protein. In these studies we find that coexpression of MDMX with E2F-1 results in degradation of the MDMX protein. Although this proteolytic degradation can be blocked by the protease inhibitors bafilomycin A₁, N-acetyl-Leu-Leu-Norleu-AL, and N-acetyl-Leu-Leu-Met-AL, MDMX degradation is not inhibited by lactacystin, suggesting that degradation occurs through a proteasome-independent mechanism. Using an E2F-1 deletion mutant (E2F-1(180–437)) we show that E2F-1-targeted degradation of MDMX does not require the E2F-1 DNA binding domain and is therefore independent of E2F-1-driven transcription. We also find that this transcriptionally inactive E2F-1 mutant is capable of degrading the MDMX-related protein MDM2 and the MDMX isoform MDMX-S. Mapping of the E2F-1 C terminus reveals that neither a previously characterized C-terminal MDM2 binding domain nor the pRb binding domain on E2F-1 is required for MDMX and MDM2 degradation.

The E2F-1 transcription factor is a regulator of both cell cycle progression and apoptosis. E2F-1 controls cell cycle transits in part by regulating transcription of genes whose products are involved in DNA synthesis. E2F-1 accomplishes this by binding DNA as a heterodimer with a Dp family member (1, 2). The ability of E2F-1 to regulate transcription is in turn controlled by the retinoblastoma protein (pRb), which inhibits E2F-1 activity by binding to the C-terminal transactivation domain of E2F-1 (3–5). Phosphorylation of pRb by cyclin D-dependent kinase is a defining event of G₁/S checkpoint progression because it results in the dissociation of pRb from the E2F-1-Dp-1 complex, allowing E2F-1 to activate transcription of its target genes.

Early studies indicated that both E2F-1-mediated apoptosis and cell cycle progression were dependent on E2F-1 transcriptional activity. Interestingly, activation of apoptosis is unique to E2F-1 among the E2F family members (6). E2F-1-driven apoptosis occurs by multiple mechanisms including both p53-dependent and -independent pathways (7, 8). Evidence suggests a requirement for the E2F-1 DNA binding domain for E2F-1-activated apoptosis (9). Activation of apoptosis by E2F-1 occurs in part through the transcriptional induction of both the p14/ARF and p73 genes (10, 11). The p73 protein, a p53 homolog, activates programed cell death in a manner analogous to p53, through the transactivation of apoptotic genes (12). The p14/ARF tumor suppressor enhances apoptosis through the p53 pathway by increasing protein degradation of MDM2, the major p53 antagonist, thereby elevating p53 levels and leading to apoptosis (13–15).

Although E2F-1 can reduce MDM2 protein levels and function, MDM2 can also affect E2F-1 function. MDM2 binds the C terminus of E2F-1 and modulates E2F-1 transcriptional activity (16, 17). MDM2 expression in cells has also been shown recently to result in a decrease in E2F-1 protein levels (18). Thus by reducing E2F-1 protein levels, MDM2 can inhibit E2F-1-driven apoptosis. In addition to its role as a negative regulator of E2F-1, MDM2 also antagonizes the p53 tumor suppressor (19). MDM2 inhibits p53-driven transcription by multiple mechanisms, including direct binding and blocking of the p53 transactivation domain (20, 21). Additionally, MDM2 targets p53 for degradation in the proteasome by functioning as an E3 ubiquitin ligase specific for p53 (22, 23). As a result of this ability to inhibit p53, MDM2 plays a role in promoting transformation (24). Consistent with this notion, increased MDM2 protein levels have been observed in a broad spectrum of human tumors (25).

The MDM2-related protein MDMX shares several of the same structural and functional properties of MDM2, including a highly conserved p53 binding domain capable of binding and inhibiting the transactivation domain of p53 (26, 27). Although MDMX can bind p53 and block its ability to activate transcription and induce apoptosis, MDMX protein can also increase the stability of p53 protein by binding MDM2 through a conserved C-terminal RING finger domain (28). The MDMX-MDM2 interaction has been shown to inhibit MDM2-targeted degradation of p53 protein (29). For example, binding of MDMX by MDM2 significantly increases the stability of the MDM2 protein with no change in MDMX stability (30, 31). Although the MDMX-MDM2 interaction has led to recent insights into the autoregulation of the MDM family of proteins, it is not currently known what effect MDMX has on E2F-1 function and vice versa. Here we show that E2F-1 can significantly decrease the steady-state levels of the MDMX protein and that the

* This work was supported in part by National Institutes of Health Grant CA67032 (to D. J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the Foerderer Foundation.

§ To whom correspondence should be addressed: Cartilage Biology and Orthopedics Branch, NIAMS, National Institutes of Health, Bldg. 13, Rm. 3W17, 13 South Dr., MSC 5755, Bethesda, MD 20892-5755. Tel.: 301-451-6860; Fax: 301-480-4325.

1 The abbreviations used are: ARF, alternative reading frame protein; OS, osteosarcoma; ALLN, N-acetyl-Leu-Leu-Norleu-AL; ALLM, N-acetyl-Leu-Leu-Met-AL; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; Ab, antibody; GST, glutathione S-transferase.
MATERIALS AND METHODS

Cells Lines, Transfections, Reporter Assays, and Protease Inhibitor Treatment—Phoenix, 293T, Ssn2, and U-2 OS cells were maintained at 37 °C under 4% CO₂ and grown in Dulbecco’s modified Eagle’s medium (BioWhitaker) supplemented with 10% fetal calf serum (Atlanta Biological). Phoenix cells are a derivative of 293 cells which have a very high efficiency of transfection (80–90%) by standard calcium phosphate methods.

24 h after transfection, Phoenix and 293T cells were split 1:7 by the calcium phosphate method with a Profection kit (Promega). 24 h after transfection, confluent U-2 OS cells were split 1:4 with Effectene transfection reagent (Qiagen). Cells were then harvested 48 h after transfection.

Extracts for the luciferase reporter assay were generated by resuspending cells in 0.25 M Tris (pH 7.8) and then sequentially freezing and thawing them in a 37 °C water bath for three cycles. Cell debris was pelleted by centrifugation at 14,000 × g for 5 min. Protein concentrations for each sample were determined using the Bradford method (Bio-Rad). Equal protein concentrations from each sample were measured for luciferase activity using luciferase assay reagent (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

The for the protease inhibitor experiments, cells were washed with phosphate-buffered saline 24 h after transfection and replaced with 10% fetal calf serum and Dulbecco’s modified Eagle’s medium containing its respected protease inhibitor. N-Acetyl-Leu-Leu-Norleu-AL (ALLN; Sigma) and N-acetyl-Leu-Leu-Met-AL (ALLM; Sigma) were used at a final concentration of 1.25 μg/ml. Baflomycin A₁ (Sigma) was used at a final concentration of 50 ng/ml (Calbiochem) at a concentration of 20 μM, zVAD-FMK (Enzyme Systems Inc.) at a concentration of 20 μM, and lactacystin (Calbiochem) was used at a final concentration of 10 μM.

Plasmids and Mutagenesis—The ARF promoter-reporter plasmid E1b-lucerase was generously provided by Karen Vousden, the MDMX-expressing plasmid was a generous gift from Edward Mercer, pCMV-pRb was provided by Antonio Giordano, and the Dp-1 expression plasmid was kindly provided by Dr. Kristian Helin. The ZF87/MAZ (32) and pMDMX- and MDMX-S-expressing plasmids have been described previously (33).

pcDNA3 expression plasmids (Invitrogen) containing epitope-tagged E2F-1(180–437), E2F-1(1–415), and MDMX114 deletion mutants were constructed by PCR amplification of the desired regions of each cDNA. Each cDNA was then cloned 3’ to a FLAG epitope tag sequence within the Bluescript KS plasmid. The FLAG epitope containing the desired cDNA within the same reading frame was then subcloned into the pcDNA3 plasmid downstream of the CMV promoter.

C-terminal (E2F-1(180–437)DF-GG) double point mutant was done by site-directed mutagenesis using pRC-CMV-E2F-1(180–437) as template. Two purified oligonucleotides (5’-GCATTGTCGGAGGAGGCGCTCCCACAGCTAGC-3’ and 5’-GGGAGGAGGCGCTCCCACAGCTAGC-3’), containing the desired mutations, were hybridized to the template and extended using Pfu polymerase (Stratagene). This reaction was repeated for 15 cycles, then template DNA was digested by incubation with the DpnI restriction enzyme for 1 h. E2F-1(180–437) and E2F-1(1–415) deletion mutants were created by amplification of the desired regions by PCR.

Generation of Extracts, Antibodies, and Immunoblotting—To generate extracts, cells were harvested by scraping in cold phosphate-buffered saline and then spun at 1,200 rpm for 5 min at 4 °C. Cytosolic extracts were generated by lysing the cells on ice in 0.1% Nonidet P-40, 10 mM Tris (pH 7.9), 1 mM phenylmethylsulfonyl fluoride, 1 μM benzamidine, 2 μM leupeptin, and 1 μM pepstatin for 10 min on ice and then centrifuged at 14,000 × g for 10 min at 4 °C and the supernatant was termed cytosol. The nuclei were resuspended in extraction buffer consisting of 0.2 M NaCl, 20 mM Hepes (pH 7.9), 20% glycerol, 0.5 μM phenylmethylsulfonyl fluoride, 1 μM benzamidine, 2 μM leupeptin, and 1 μM pepstatin for 10 min on ice and then centrifuged at 14,000 × g for 8 min to pellet the residual nuclear material. The supernatant from this centrifugation spin was termed nuclear extract.

Equal quantities of protein extracts were separated by size using SDS-polyacrylamide gel electrophoresis. Proteins were then transferred electrophoretically onto nitrocellulose (Bio-Rad) and blocked for 1 h at 37 °C prior to induction of GST expression with 0.5 μM isopropyl-1-thio-β-D-galactopyranoside. After

30 min in 5% dry milk and resuspended in TBST buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20). The nitrocellulose blots were incubated in primary antibody for 1 h on a shaker at room temperature. The blots were washed three times with TBST for 5 min and then incubated in secondary antibody on a shaker for 30 min at room temperature. The blots were washed in TBST again three times for 5 min each wash on a shaker at room temperature. Blots were developed using the Renaissance (PerkinElmer Life Sciences) chemiluminescent reagents and X-Omat Blue XB-1 film (PerkinElmer Life Sciences).

Primary antibodies for immunoblot analysis were diluted in 3% dry milk and TBST at the following ratios: 1:2,000 anti-E2F-1 Ab-1 (Calbiochem), 1:5,000 anti-DP-1 M2 (Ab-1) (Calbiochem), 1:2,000 anti-Dp-1 K-20 (Santa Cruz Biotechnology), 1:2,000 anti-pRb C-15 (Santa Cruz Biotechnology), and 1:2,000 anti-BRCA1 Ab-1 (Oncogene Research Products). Horseradish peroxidase-conjugated anti-rabbit Ig (Amersham Pharmacia Biotech) and horseradish peroxidase-conjugated anti-mouse Ig (Amersham Pharmacia Biotech) secondary antibodies were diluted 1:10,000 in 3% dry milk and TBST.

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-labeled methionine in T7 Quick (Promega) rabbit reticulocyte lysate for 1 h at 30 °C. GST-Dp-1 was created by amplifying the desired cDNAs by PCR and 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 Escherichia coli. Bacteria cultures were transformed with the appropriate pGEX expression plasmid grown under ampicillin medium at 37 °C overnight. The transformed bacteria culture was then diluted 1:10 in selective medium and grown for 1 h at 37 °C prior to induction of GST expression with 0.5 μM isopropyl-1-thio-β-D-galactopyranoside. After
E2F-1 Reduces the Steady-state Levels of the MDMX Protein—In an effort to identify E2F-1-associated proteins, we set up a two-hybrid yeast screen using portions of E2F-1 as bait (linked to LexA). We isolated a number of cDNAs whose products appear to interact with E2F-1 in yeast. One positive clone from this screen was the MDMX protein, an MDM2-related product. We could demonstrate a clear interaction between E2F-1 and MDMX in an in vitro assay. This is shown in the GST pull-down assay in Fig. 1A. From the figure it is clear that MDMX associates well with E2F-1 but not with GST alone or with GST-Dp1. However, attempts to show an in vivo interaction between E2F-1 and MDMX were unsuccessful, as assessed by coimmunoprecipitation experiments in extracts of cells transfected with E2F-1 and MDMX expression vectors. Interestingly, Western blot analysis of these same extracts revealed a significant reduction in MDMX protein when coexpressed with E2F-1. As shown in Fig. 1B, ectopically expressed MDMX protein is easily detectable in cell extracts by Western blot analysis after transient transfection of the MDMX expression vector. The upper band is a normal product of MDMX and may represent a modified form of the protein. Interestingly, when MDMX is coexpressed with E2F-1 protein, the steady-state level of MDMX was diminished markedly, but the level of E2F-1 was unaffected (Fig. 1B). These data indicate that E2F-1 expression appears to reduce the level of the MDMX protein.

The Decrease in the MDMX Protein by E2F-1 Is Independent of E2F-1-driven Transcription—It has been shown previously that the E2F-1 transcription factor targets the degradation of the MDM2 protein (14). This is likely because E2F-1 transcriptionally activates the p14/ARF gene, whose protein product can in turn target the destruction of MDM2 by the proteasome. It is possible that E2F-1 could similarly be directing the breakdown of MDMX by the transactivation of the p14/ARF gene. To test whether E2F-1-mediated reduction in the MDMX protein is dependent on E2F-1-driven transcription, an N-terminal deletion mutant of E2F-1, termed E2F-1(180–437), was created which lacks the DNA binding domain (Fig. 2A) yet retains the activation domain and the pRb binding domain. Fig. 2B demonstrates that the N-terminal 179 amino acids of E2F-1 are essential for transcriptional activation of the p14/ARF promoter in a transient transfection assay. Although full-length E2F-1 is capable of enhancing the activation of a luciferase reporter construct driven by the ARF promoter (by more than 10-fold), E2F-1(180–437) is completely ineffective at activating the ARF promoter. Interestingly, E2F-1(180–437) is capable of decreasing the amount of coexpressed MDMX protein significantly, to an extent similar to that of the full-length E2F-1 protein (Fig. 2C). These data indicate that the E2F-1-mediated reduction in MDMX protein is independent of E2F-1-driven transcription and activation of the ARF promoter. These data also indicate that only the C-terminal half of the E2F-1 protein is required for the reduction in MDMX protein levels.

Dp-1 and pRb Are Not Targeted for Degradation by E2F-1, nor Do They Block E2F-1-targeted Reduction of MDMX—To determine whether the E2F-1-induced reduction in steady-state MDMX protein is specific to the MDMX protein, we tested to see if E2F-1(180–437) could alter the levels of the E2F-1-binding proteins Dp-1, pRb, the ZF87/MAZ transcription factor (34), or the BRCA1 protein. Ectopically expressed ZF87/MAZ, pRb, BRCA1, and Dp-1 are readily detectable in cell extracts by immunoblot assay after transient transfection of the respective expression plasmids.
expression plasmids (Fig. 3, A–D). Although E2F-1 (180–437) was capable of greatly reducing MDMX protein levels (Fig. 3A), it had no effect on the steady-state protein levels of the ZF87/MAZ, Dp-1, or pRb proteins (Fig. 3, A, B, and C). These results suggest that E2F-1-targeted reduction in MDMX protein levels may be specific for the MDMX protein. Interestingly, E2F-1 (180–437) was able to target the BRCA1 protein for degradation (see Fig. 3D). As will be discussed below, this appears consistent with a certain type of proteolytic mechanism activated by E2F-1.

From the above it appears that pRb protein levels are not affected by E2F-1. However, pRb is known to block proteasomal degradation of MDM2 by repressing E2F-1-driven transactivation of p14/ARF (14). We were interested in determining whether pRb could regulate the E2F-1 (180–437)-targeted reduction of MDMX, so we coexpressed pRb, E2F-1 (180–437), and MDMX proteins in cells and measured the relative quantity of MDMX protein by Western blot analysis (Fig. 4A). We found that coexpression of the retinoblastoma protein was unable to block E2F-1 (180–437)-targeted reduction of MDMX. The inability of pRb to block MDMX reduction is further evidence that the E2F-1 (180–437)-induced reduction of MDMX is independent of E2F-1-driven transcription.

A recent study done in Saos2 cells demonstrates that MDM2 can reduce the quantity of E2F-1 protein when the Dp-1 protein is present (18). This suggests that there is a possibility that Dp-1 dimerization may interfere with E2F-1-mediated MDMX reduction. To test whether the Dp-1 protein inhibits the MDMX protein reduction, we coexpressed E2F-1, Dp-1, and MDMX proteins in cells and measured the relative quantity of MDMX protein. As seen in Fig. 4B, Dp-1 expression was unable to inhibit the E2F-1-induced reduction of MDMX. Thus, Dp1 dimerization with E2F-1 does not affect the mechanism by which E2F-1 degrades MDMX.

E2F-1 targets the MDMX Protein for Degradation by a Proteasome-independent Pathway — We were next interested in determining whether E2F-1 was reducing MDMX protein levels by increasing MDMX degradation through a specific proteolytic pathway. We therefore tested whether several protease inhibitors could block E2F-1 (180–437)-mediated reduction of MDMX. Because the level of reduction of MDMX by E2F-1 was so great it was thought necessary to achieve more moderate levels of reduction which could be overcome by the protease inhibitor. It was first necessary therefore to reduce the amount of transfected the E2F-1 (180–437)-expressing plasmid. Fig. 5A demonstrates that there is a dose dependence to E2F-1-targeted reduction of MDMX in that as the amount of cotransfected E2F-1 plasmid is decreased the level of MDMX protein increases. We then analyzed several protease inhibitors for the ability to block the reduction of MDMX protein by a low dose of E2F-1 (180–437) expression plasmid, as shown in Fig. 5B. First, through the use of the competitive proteasome inhibitor ALLN and its proteasome-ineffective analog ALLM we determined whether there was any dependence on proteasome function (35). As seen in Fig. 5B, both ALLN and ALLM were capable of blocking E2F-1 (180–437)-targeted reduction of MDMX. Although this suggests that the reduction of steady-state MDMX by E2F-1 is caused by proteolytic degradation of the MDMX protein, it appears that this MDMX degradation is not dependent on the proteasome (note that ALLN and ALLM inhibit the action of other proteases, as discussed below). To test further the dependence of the proteasome we analyzed lactacystin for its ability to prevent E2F-1 (180–437)-targeted MDMX degradation. Lactacystin is a potent irreversible inhibitor that is highly specific for the proteasome (36). Lactacystin was unable to block the relative decrease in MDMX protein resulting from E2F-1 (180–437) expression (Fig. 5B). It is therefore likely that a proteolytic mechanism separate from the proteasome exists and that it is activated by E2F-1 and capable of degrading MDMX.
inactive E2F-1 targets MDM proteins for degradation. Panel A, graded reduction in MDMX protein by increasing amounts of E2F-1. Phoenix cells were transfected with 10 μg of MDMX expression plasmid and an increasing amount of E2F-1(180–437) expression plasmid (left to right). pcDNA3 was used to equalize the amount of DNA in transfections. The transiently expressed MDMX protein was detected by immunoblot analysis of 40-μg Phoenix extracts using an anti-FLAG antibody specific for the 5′-epitope of MDMX. The arrows indicate the positions of MDMX. Panel B, Phoenix cells were transiently transfected with either 10 μg of MDMX expression plasmid or with 10 μg of MDMX + 1 μg E2F-1(180–437) (as in panel A). Cells were then grown for 24 h in the presence of 10 mM lactacystin, 50 ng/ml bafilomycin A1, 1.25 μg/ml ALLN, 1.25 μg/ml ALLM, 20 μM PD150606, 20 μM zVAD-FMK, or no inhibitor. The cells were harvested and extracts generated, 40 μg of which was used in immunoblot using anti-FLAG M2 primary antibody. The levels of MDMX protein were arbitrarily set to 100% in the absence of E2F-1(180–437) for each treatment condition. The error bars indicate the S.E. of the mean for multiple experiments. Panel C, RT-PCR analysis of cells expressing E2F-1(180–437) in the presence or absence of MDMX. Total RNA was isolated and 1 μg used in the RT-PCRs with primers specific for MDMX and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The products were electrophoresed on agarose gels, which were stained with ethidium bromide. Shown are pictures of the stained gels; the correct products are indicated by the arrows.

As just mentioned, in addition to being a competitive inhibitor of the proteasome, ALLN is a potent inhibitor of both calpain and cathepsin-like proteases, a characteristic retained by the ALLM analog (37). We therefore tested the ability of the broad cathepsin inhibitor bafilomycin A1 (38) for its ability to block E2F-1(180–437)-targeted degradation of the MDMX protein. Similarly to ALLN and ALLM, bafilomycin A1 is able to block the E2F-1(180–437)-targeted degradation of MDMX protein (Fig. 5B). We saw no increase in the steady-state quantity of MDMX protein when we treated cells with the caspase inhibitor zVAD-FMK (39) or the calpain inhibitor PD150606 (40). These results strongly suggest that E2F-1-targeted degradation of MDMX occurs through the activation of a cathepsin-like protease.

As a control, we determined whether the E2F-1(180–437) protein could reduce relative MDMX mRNA levels. We performed RT-PCR from mRNA isolated from Phoenix cells transiently transfected with control, MDMX, E2F-1(180–437), and MDMX/E2F-1(180–437) expression plasmids. We found that the presence of E2F-1(180–437) does not reduce the steady-state levels of MDMX mRNA (Fig. 5C). These data in total suggest that the reduction in MDMX protein is the result of MDMX protein degradation and not an effect on MDMX transcript levels.

E2F-1(180–437) also targets MDM2 and the C- and N-terminal halves of MDMX for degradation—Because E2F-1 is able to affect the steady-state protein levels of MDMX, it was important to determine whether this effect included other MDM family members. It has been reported previously that MDM2 protein levels can be reduced by the expression of E2F-1 (14). As mentioned above, this may be because E2F-1 induces the transcriptional activation of p14/ARF, which targets MDM2 for degradation in the proteasome. Because it is possible that E2F-1 targets MDM2 for degradation by multiple mechanisms, we determined whether E2F-1(180–437) was capable of reducing the steady-state levels of MDM2 protein. MDM2 protein is easily detectable by immunoblot after transient transfection of cells with an MDM2 expression plasmid (Fig. 6A). Coexpression of the E2F-1(180–437) protein with MDM2 greatly reduced the quantity of MDM2 protein, similar to what has been seen for MDMX. These data indicate that a transcriptionally inactive E2F-1 is capable of reducing steady-state levels of both MDMX and MDM2. Interestingly, coexpression of the MDMX protein was unable to block the E2F-1(180–437)-mediated reduction in MDM2 protein.

Although both MDM2 and MDMX proteins are susceptible to protease degradation induced by the transcriptionally inactive E2F-1(180–437) it is not clear which regions of the MDM proteins are targeted for degradation. Therefore, to explore the specificity of E2F-1-targeted degradation of the MDM proteins, we analyzed the effects of E2F-1 on MDMX-S. MDMX-S is an isoform of MDMX, encoding a truncated MDMX protein containing only the N-terminal p53 binding domain of MDMX (shown in Fig. 6B) (33). In addition to MDMX-S, an MDMX...
N-terminal deletion mutant, termed MDMXd114 (shown in Fig. 6D), was created which has no amino acids in common with MDMX-S. The MDMX-S and MDMXd114 proteins were easily detectable in cell extracts by immunoblot in cells transiently transfected with the respective expression plasmids, as shown in Fig. 6C. However, neither protein was detectable when coexpressed with E2F-1(180–437), as shown in Fig. 6C.

Further, the addition of ALLM to the cells was able to block the reduction in both MDMX-S and MDMXd114 levels mediated by E2F-1(180–437) (data not shown). This suggests that both the MDMX-S and MDMXd114 proteins are degraded in a manner similar to that of full-length MDMX. This result also demonstrates that there likely are multiple domains within the MDMX protein which are targeted for degradation by this E2F-1-mediated mechanism.

As a control in these experiments, we performed RT-PCR assays on mRNA isolated from cells transfected with the control, MDMX-S, MDMXd114, E2F-1(180–437), MDMX-S/E2F-1(180–437), and MDMXd114/E2F-1(180–437) expression plasmids. We found that the presence of E2F-1(180–437) does not reduce the steady-state levels of MDMX-S or MDMXd114 mRNA (Fig. 6D). These data indicate that E2F-1 does not affect transcript levels of MDMX and MDMXd114.

The E2F-1 C Terminus Does Not Play a Role in the Degradation of MDMX—From the data presented above it is clear that the E2F-1(180–437) mutant, which does not contain the DNA binding domain (41) or the cyclin A/cdk2 binding domain (42), is sufficient for targeting the MDM proteins for proteolytic degradation. However, the E2F-1(180–437) mutant does contain a transcriptional transactivation domain within its C terminus which mediates an interaction with a number of cellular proteins (e.g. tata-binding factor, CBP, TRRAP, and TFIIFH). We therefore generated the C-terminal deletion mutant E2F-1(1–415), lacking residues 415–437 (shown in Fig. 7A), which are necessary for activation of transcription and pRb binding (43, 44). This mutant, which was severely deficient in activating transcription from the p14/ARF promoter (data not shown), was then tested for its ability to degrade the MDMX protein. In a cotransfection experiment E2F-1(1–415) was sufficient to reduce the steady-state levels of MDMX protein, as shown in Fig. 7B. These data, along with the pRb coexpression data in Fig. 4A, reveal that the transactivation domain, which encompasses the pRb binding domain, is not essential for directing the degradation of MDMX by this proteolytic mechanism.

The C terminus of E2F-1 has also been shown to be involved in an interaction between E2F-1 and MDM2 (16). A double point mutation within E2F-1 of both aspartic acid 390 to glycine and phenylalanine 391 to glycine has been reported to disrupt the MDM2-E2F-1 interaction (16). We therefore created an E2F-1 mutant with these amino acid substitutions (termed E2F-1(180–437)(DF-GG)), as shown in Fig. 7A. Interestingly, the E2F-1 mutant deficient in binding to MDM2 was still capable of reducing the steady-state levels of both MDM2 and MDMX protein, in cotransfection experiments, as shown in Fig. 7, C and D. These data show that the amino acid residues in E2F-1 required for its high affinity association with MDM2 are not required for degradation of either MDM2 or MDMX.

E2F-1-targeted Proteolytic Degradation of MDMs Is Not Spe-
sific for Phoenix Cells—As a control in these experiments we also tested other cell lines for the ability of E2F-1 to reduce steady-state MDMX protein levels. We found that E2F-1 targeted the degradation of MDMX in the parental 293T cell line and in U-2 OS cells (Fig. 8), as well as in Saos2 cells (data not shown). These data indicate that the mechanism of MDM degradation is likely not cell type-specific.

**DISCUSSION**

In this study we provide evidence that the E2F-1 protein is capable of targeting the MDM2, MDMX, and MDM5 proteins for proteolytic degradation. By transiently coexpressing E2F-1 and the MDM proteins, we see a clear reduction in the levels of the MDM proteins. However, we show that E2F-1 does not reduce the levels of other coexpressed proteins such as ZF8/MAZ, Dp-1, or pRb, so there may be some specificity to this effect. We also find that the reduction in MDMX protein resulting from E2F-1 is not caused by a decrease in MDMX mRNA, indicating that the MDMX protein itself is the target. Further, protease inhibitors added to the cells are able to block the reduction in MDMX levels by E2F-1. These data reveal that one function of E2F-1 is the activation of the proteolytic degradation of the MDM proteins. E2F-1 has been shown previously to target the degradation of the MDM2 protein (14), the result in part of the ability of E2F-1 to activate transcriptionally p14/ARF, a protein that...
Inactive E2F-1 Targets MDM Proteins for Degradation

In the presence of the E2F-1 DNA binding domain, and therefore the E2F-1-induced degradation of the MDM proteins in this system is independent of E2F-1-driven transcription. Thus, these appear to be at least two mechanisms for E2F-1-induced MDM protein degradation: one that targets MDM degradation through the transcriptional activation of p14/ARF (14) and one that targets independently of E2F-1-driven transcription.

The Mechanism of E2F-1-targeted Degradation of the MDM Family of Proteins—The E2F-1-activated MDM degradation that we describe here, by the mechanism independent of E2F-1-driven transcription, also appears independent of proteasome. We show that a proteasome inhibitor does not interfere with the ability of E2F-1(180–437) to reduce MDMX levels. This suggests that a separate protease is responsible for the degradation of MDMX, which would be induced by the expression of E2F-1 in this system. We also find that protease inhibitors that are able to block the function of cathepsin type proteases are capable of blocking the reduction of MDMX caused by E2F-1(180–437) expression, suggesting that the protease involved may be cathepsin-like.

We also tested several protease inhibitors for their ability to inhibit E2F-1(180–437)-targeted reduction of MDMX protein. The protease inhibitors ALLN and ALLM blocked the decrease in MDMX protein caused by the coexpression of E2F-1(180–437). ALLN is a competitive inhibitor of the proteasome and the cathepsin and calpain type proteases, whereas ALLM is an effective inhibitor against only calpain and cathepsin proteases. We believe that this suggests that E2F-1(180–437) causes the degradation of MDMX by a mechanism independent of the proteasome. This hypothesis was supported by the finding that the specific irreversible proteasome inhibitor lactacystin was unable to block E2F-1(180–437)-induced degradation of MDMX.

In addition to ALLN and ALLM, we found that bafilomycin A1 also inhibited E2F-1(180–437)-targeted reduction in MDMX protein. Bafilomycin A1 is an inhibitor of lysosomal H^+-ATPase (38) and therefore also inhibits lysosomal cathepsin proteases similar to ALLN and ALLM. Although it is more common for nuclear proteins, such as transcription factors, to be targeted for degradation in the proteasome, there have been some reported exceptions. A protease termed SPase has been identified which has specificity for degrading both the Sp1 transcription factor and pRB (45). Also, a cathepsin-like protease localized to the nucleus has been shown to be involved in the turnover of the BRCA1 protein in some cell types (46). These proteases may have overlapping characteristics with the protease involved in E2F-1-targeted degradation because BRCA1 also appears to be targeted for destruction by E2F-1, as shown above in Fig. 3D.

Additional evidence to support the finding that the mechanism of MDMX reduction by E2F-1 is independent of E2F-1-driven transcription is the regulation of these pathways by the retinoblastoma tumor suppressor pRb. The pRb protein is known to bind and block the transactivation domain of E2F-1, thereby preventing E2F-1-activation of the p14/ARF promoter (14). pRb is therefore capable of blocking this form of E2F-1-targeted degradation of MDM2. Evidence suggests that this pathway links together several known tumor suppressors and oncopgenes, including pRB and p53 (8). Here we have shown that coexpression of pRB does not interfere with E2F-1(180–437)-targeted degradation of the MDM proteins. Also, we demonstrate that E2F-1(1–415), which is not capable of binding pRB, maintains the ability to degrade MDMX proteins. Therefore, this mechanism appears to be independent of pRB function.

The exact mechanism by which E2F-1 targets MDM family members for degradation is still unknown. Further, it is not yet known whether it is a direct or indirect mechanism. Given that E2F-1 and MDMX (and MDM2) interact in vitro and in vivo, it is possible that E2F-1 associates directly with MDMX, for example, and then targets it for destruction. However, the fact that E2F-1 targets MDMX for destruction makes it problematic to show a direct interaction of the two proteins in mammalian cells (e.g. by coimmunoprecipitation). In terms of mechanisms, it is also possible that E2F-1 activates a separate cathepsin-like protease that specifically targets the MDM proteins for destruction. Such an indirect mechanism would not require a physical interaction between E2F-1 and MDMX. It will now be important to determine the nature of the mechanism by which E2F-1 targets MDMX for destruction.

The Biological Role of E2F-1-mediated Breakdown of the MDM Proteins—As outlined above, E2F-1 is a transcription factor that is known to activate apoptosis. This is accomplished in part by the ability of E2F-1 to transactivate transcriptionally the p14/ARF gene, which results in the targeted destruction of MDM2 in the proteasome (by p14/ARF) and the induction of apoptosis (14). In fact, the targeted reduction of the MDM2 protein as a result of E2F-1 expression has been observed during E2F-1-activated apoptosis (47). It remains to be determined, however, whether the proteasome-independent E2F-1-targeted degradation of MDM proteins in the work presented here may also play a role in E2F-1-activated apoptosis. However, it is possible that the E2F-1-targeted reduction of the MDM proteins could function either to activate apoptosis directly or to increase the sensitivity of a cell to apoptosis. In this regard we are examining the sensitivity of cells expressing E2F-1/MDMX to DNA-damaging agents to determine whether MDMX counters the effects of E2F-1 in chemosensitivity. It would certainly appear that E2F-1 and the MDM proteins have opposing effects on the induction of apoptosis. If this is true, the MDM family will be crucial regulators of E2F-1 function and vice versa.

Acknowledgments—We thank Ed Mercer for the MDM2 expression plasmid, Antonio Giordano for the pCMV-pRb, Karen Vousten for the ARF reporter (E1β-luciferase), and Kristian Helin for the Dp-1 expression plasmid.
A Transcriptionally Inactive E2F-1 Targets the MDM Family of Proteins for Proteolytic Degradation
Gordon D. Strachan, Ravikumar Rallapalli, Bruna Pucci, Toulouse P. Lafond and David J. Hall

J. Biol. Chem. 2001, 276:45677-45685.
doi: 10.1074/jbc.M103765200 originally published online September 21, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103765200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 22 of which can be accessed free at http://www.jbc.org/content/276/49/45677.full.html#ref-list-1