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E2F1 Induces Cell Death, Calpain Activation, and MDMX Degradation in a Transcription Independent Manner Implicating a Novel Role for E2F1 in Neuronal Loss in SIV Encephalitis

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Abstract The E2F1 transcription factor can initiate proliferation or apoptosis, the latter by both transcription-dependent and -independent mechanisms. Recently, an E2F1 mutant lacking the DNA binding domain, E2F1(180–437), has been implicated in degradation of MDMX and MDM2 proteins via lysosomal proteases. MDM proteins block p53 dependent apoptosis by directly inhibiting p53 stability and function. Here we demonstrate E2F1(180–437) induces death in HEK293 cells independent of E2F1 transcriptional activation and p53 stabilization. E2F1(180–437) elevates the activity of the calcium-activated protease, calpain, which is required for E2F1 induced proteolysis of MDMX and E2F1 induced cell loss. To determine if E2F1 could be activating proteolysis via calpains in neurodegeneration, we examined MDMX immunofluorescence in simian immunodeficiency virus encephalitis (SIVE). We found a reciprocal relationship between E2F1 and MDMX staining: in SIVE where E2F1 immunostaining is increased, MDMX is decreased, while in controls where E2F1 immunostaining is low, MDMX is high. Together these experiments support a new function for E2F1 in the activation of calpain proteases and suggest a role for this pathway in SIVE. J. Cell. Biochem. 96: 728–740, 2005. © 2005 Wiley-Liss, Inc.

Key words: necrosis; apoptosis; E2F1; SIV; encephalitis; cell cycle; neurodegeneration; calpain; protease; lysosome

The E2F family (E2F1–E2F6), is responsible for regulating the transcriptional expression of several genes important for cell cycle progression and DNA synthesis [Black and Azizkhan-Clifford, 1999]. When E2F proteins are bound by a member of the pocket protein family, such as the retinoblastoma protein, their transactivating activity is repressed, arresting cells at the G1/S cell cycle checkpoint [Adams and Kaelin, 1996; Nevins et al., 1997]. Dissociation of pocket proteins from E2Fs by cyclin-dependent kinase phosphorylation is the hallmark of G1/S checkpoint passage and allows for progression through S phase, propelled by E2F-driven transcription [Zetterberg et al., 1995].

E2F1 is unique among the E2F family of proteins in its ability to induce cell death, which occurs though several mechanisms [Ginsberg, 2002; Hallstrom and Nevins, 2003]. The best characterized pathway by which E2F1 activates cell death involves direct transcriptional activation of pro-apoptotic genes such as p19/ARF [Zhu et al., 1999], Apaf-1 [Moroni et al., 2001], and p73 [Stiewe and Putzer, 2000]. Activation of p19/ARF targets the anti-apoptotic protein, MDM2, for degradation in the proteasome. MDM2 prevents apoptosis induced by the p53 tumor suppressor protein by targeting p53 for

Abbreviations used: Apaf-1, apoptosis protease-activating factor 1; CDK, cyclin dependent kinase; GFP, green fluorescent protein; HIVE-human immunodeficiency virus encephalitis; SIVE, simian immunodeficiency virus encephalitis.

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proteosomal degradation [Michael and Oren, 2003]. In the absence of MDM2, p53 is stabilized and can induce cell cycle arrest and/or apoptosis [Honda et al., 1997]. By increasing expression of Apaf-1, apoptosis protease-activating factor 1, E2F1 activates apoptosis by increasing activity of the apoptosome and inducing death independent of the p53 proteins [Cain et al., 2002]. The p73 protein is a member of the p53 family of transcription factors which transactivates known apoptotic genes [Melino et al., 2003]. E2F1 has also been shown to induce apoptosis by targeting the degradation of the TRAF2 protein, a known inhibitor of apoptosis that blocks caspase 8 activation [Phillips et al., 1999]. Interestingly, all of these mechanisms of E2F1-activated apoptosis require the presence of a functional E2F1 DNA binding domain and in addition require that E2F1 be targeted to the nucleus [Hsieh et al., 1997].

E2F1 has been implicated in neuronal cell death by mechanisms independent of both p53 and caspase 3 activity. Cortical neurons lacking E2F1 are resistant to beta amyloid toxicity, a model for Alzheimer's disease [Giovanni et al., 2000]. This mode of death does not require p53 or caspase 3 suggesting E2F1 induced death may be different in neurons than other cell types. Similarly, apoptosis of cerebellar granule neurons induced by dopamine [Hou et al., 2001], K^+ deprivation [O'Hare et al., 2000], and trophic factor withdrawal [Liu and Greene, 2001] also require E2F1. Finally, overexpression of E2F1 in cortical neurons induces apoptosis [O'Hare et al., 2000], suggesting that E2F1 is necessary and sufficient for neuronal cell death.

In vivo findings suggest that neuronal loss during neurodegenerative diseases may be regulated by E2F1. Immunohistochemical analysis indicates that the E2F1 protein is increased in the neuronal cytoplasm of involved brain regions in HIV encephalitis (HIVE), simian-immunodeficiency virus encephalitis (SIVE) [Jordan-Sciutto et al., 2000a,b, 2002a,b], Alzheimer's Disease [Jordan-Sciutto et al., 2002a,b], and amyotrophic lateral sclerosis [Ranganathan and Bowser, 2003] as compared to unaffected, age-matched control brain tissue. Interestingly, in all of these diseases, increased expression of E2F1 is localized to the cytoplasm suggesting either inhibition of E2F1 function or an alternate function for E2F1 in the cytoplasm that has not yet been described.

We have recently shown that expression of an E2F1 mutant, E2F1(180–437), which lacks the basic-helix-loop-helix DNA binding domain, results in the proteolytic degradation of the MDM2, MDMX, and MDMX-S proteins [Strachan et al., 2001]. This proteolytic degradation of the MDM proteins occurs by a cathepsin-like protease independent of proteosome function. Since E2F1(180–437) also lacks a nuclear localization signal [Muller et al., 1997], this E2F1 mutant localizes predominantly to the cytoplasmic compartment. Thus, this mutant may serve as a model for neuronal loss in neurodegenerative diseases.

While known mechanisms of E2F1-induced cell death require a DNA binding domain, degradation of the anti-apoptotic MDM proteins by E2F1(180–437) would suggest a mechanism for promoting cell death independent of E2F-driven transcription. This has led us to hypothesize that E2F1 induces death by a novel pathway dependent on activation of proteases and subsequent degradation of MDM proteins. Here we show that E2F1(180–437) reduces cellular viability independent of p53. Further, cytoplasmic E2F1-induced death is mediated by activation of the calpain family of proteases. Finally, evidence of E2F1-induced calpain activation is seen in neurons of macaques with SIVE suggesting this death pathway may be activated in neurodegenerative diseases associated with neuroinflammation.

MATERIALS AND METHODS

Plasmids and Transfections

The ARF reporter plasmid, E1β-luciferase was generously provided by Dr. Karen Vousden, expression plasmids expressing MDM2 and p53 were a generous gift from Dr. Edward Mercer, the calpastatin gene from the Dr. Masatoshi Maki laboratory was subcloned into the pCDNA3.1+ expression plasmid and kindly provided by Dr. Edward Lally and the Us9-EGFP expression plasmid (pBB14) was kindly provided by Dr. L.W. Enquist. pCDNA3 expression plasmids (Invitrogen) containing epitope tagged MDMX, MDMX-S, E2F1, and E2F1(180–437) proteins have been described previously [Strachan et al., 2001].

293T cells were maintained at 37°C under 4% CO_2 and grown in DMEM (BioWhittaker) supplemented with 10% fetal calf serum (Atlanta Biological). Cells were split 1:7 from confluence,

24 h prior to transfection. The 293T cells were then transfected by the calcium phosphate method using the Profection kit (Promega).

Western Blot

Cells were harvested by scraping in cold PBS, and then spun at 1200 RPM for 5 min at 4°C. Extracts were generated by lysing cells on ice in 0.1% Nonidet P-40, 10 mM Tris (pH 7.9), 10 mM $MgCl_2$, 15 mM NaCl, and the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (1 μg/ml), pepstatin (2 μg/ml), and leupeptin (1 μg/ml). Extracts were centrifuged at 800*g* for 10 min, and the soluble faction was used in Western blot experiments.

Protein concentrations were determined using the Bradford method (Biorad), and then equal quantities of protein were separated using a NuPage 4%–12% Bis-Tris gel (Invitrogen). Proteins were then electophoretically transferred onto nitrocellulose (Biorad) then blocked for 30 min in 2% BSA in TBST buffer [10 mM Tris (pH 8); 150 mM NaCl; 0.05% Tween 20]. Blots were incubated in primary antibody for 1 h 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 finally developed using the Renaissance (NEN Life Sciences) chemiluminescent reagents and X-omat Blue XB-1 film (NEN Life Sciences).

Primary antibodies for Western blot analysis were diluted in TBST at the following ratios—1:5,000 for the monoclonal M2 anti-FLAG (Kodak), 1:1,000 for the monoclonal Ab-1 anti-MDM2 (Calbiochem), and 1:3,000 for the monoclonal Ab-1 anti-p53 (Oncogene Science, Inc.). The polyclonal A38 anti-calpain-cleaved spectrin antibody was a kind gift of Dr. Robert Siman, and was used at a concentration of 1:15,000. HRP conjugated anti-mouse Ig and HRP conjugated anti-rabbit IgG (Amersham Life Science) secondary antibody were diluted 1:10,000 in TBST.

Reporter Assays

Extracts for the luciferase and β-galactosidase reporter assays were generated by resuspending cells in 100 mM Tris (pH 7.8) by sequentially freezing the cells on dry ice then thawing 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 (Biorad). Equal protein concentrations from each sample were measured for luciferase activity using Luciferase Assay Reagent (Promega) and a Monolight 2010 Luminometer (Analytical Luminescence Laboratory).

Cell Sorting and FACS Analysis

Phoenix cells were transfected with a Us9-EGFP expression plasmid and either control (pcDNA3), E2F1 or E2F1(180–437) expression plasmids in a 1:3 ratio, respectively. Cells were allowed to grow for 48 h following transfection, then were from tissue culture plates by trypsinization and resuspended in PBS. Cells were sorted based on green fluorescence using a Coulter EPICS Elite (Beckman Coulter, Inc.) cell sorter. Green fluorescent "positive" and "negative" cells were sorted into PBS then replated in 10% fetal calf serum for an additional 72 h. Cells were then trypsinized, washed in PBS, then fixed in 70% ethanol/PBS for 20 min on ice. Cells were again washed in PBS, then treated with 180 μg/ml of Rnase A (Boehringer Ingelheim) for 20 min at room temperature. Propidium iodide was added to each sample to a final concentration of 50 μg/ml and incubated for 15 min at room temperature before analyzing 20,000 cells from each sample on a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc.) for DNA content.

SIV Tissue

Rhesus macaques were housed and maintained according to strict Association for Assessment and Accreditation of Laboratory Animal Care standards. Macaques were derived from vaccine trials, infected with SIVdeltaB670, and killed at variable times after infection. SIVE was empirically defined as the presence of abundant perivascular mononuclear infiltrate and microglial nodules in necropsied brain tissues. Multinucleated giant cells were present in some lesions of all SIVE cases. Staining for E2F1 and MDMX was assessed in four non-infected, non-encephalitic cases, five SIV infected, non-encephalitic cases, and three cases with SIV encephalitis.

Immunohistochemistry and Immunofluorescence

Paraffin-embedded sections were heated to 50°C for at least 30 min, and deparaffinized in

Histoclear (15 min, three times) (National Diagnostics, Atlanta, GA). Sections were rehydrated as follows: 100% alcohol for 10 min, two times; 95% alcohol for 10 min; 90% alcohol for 10 min; 70% alcohol for 10 min; H_2O for 5 min. Endogenous peroxidase activity was inactivated by immersing in 3% H_2O_2 in Methanol for 30 min. Antigen unmasking was performed by placing slides in target retrieval solution (Dako, Carpinteria, CA) at 95°C for 1 h. After gradual cooling to room temperature, tissue sections were blocked with 10% normal goat serum in PBS. Antibodies to E2F1(KH95, monoclonal) and MDMX(Ab-1, monoclonal) (Santa Cruz Biotechnology, Santa Cruz, CA), were used at dilutions defined empirically for each lot (around 1:100 for E2F1 and 1:1,000 for MDMX). The tyramide amplification system (New England Biolabs) was used to detect E2F1 as previously described [Jordan-Sciutto et al., 2000a,b]. DNA staining was visualized by DAPI staining. DAPI was used at 0.25 ng/ml and incubated on the slides for 30 min. Immunofluorescent slides were mounted in gelvatol [Ausubel et al., 1994] and analyzed by laser confocal microscopy on a Biorad Radiance 2100 equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (Biorad), as described previously [Strachan et al., 2003]. All images shown in the figures were captured with uniform threshold settings.

Immunocytochemical staining was performed using a Vector® Nova-Red detection system (Vector). Slides were counterstained with hematoxylin, dehydrated in graded alcohol, and coverslipped with permount.

Statistical Analysis

For experiments involving quantification, an average of three experiments is shown with a standard deviation indicated by an error bar. A two-tailed Student T-test was used to determine if two experimental conditions differed significantly.

RESULTS

E2F1 Induces Cell Death Independent of DNA Binding and Transcriptional Activation

We have previously observed that expression of an E2F1 mutant, E2F1(180–437), lacking the N-terminal 180 amino acids, results in the proteolytic degradation of the MDM2, MDMX, and MDMX-S proteins in cell culture [Strachan et al., 2001]. This led us to hypothesize that E2F1-mediated reduction of the anti-apoptotic MDM proteins would lead to activation of cell death. To test our hypothesis, we first reproduced our initial observations in HEK 293T cells. When E2F1(180–437) was overexpressed with MDM2, MDMX, and MDMX-S in HEK293T cells, we observed reduced levels of MDM2, MDMX, and MDMX-S proteins 48 h following transfection by immunoblot (Fig. 1).

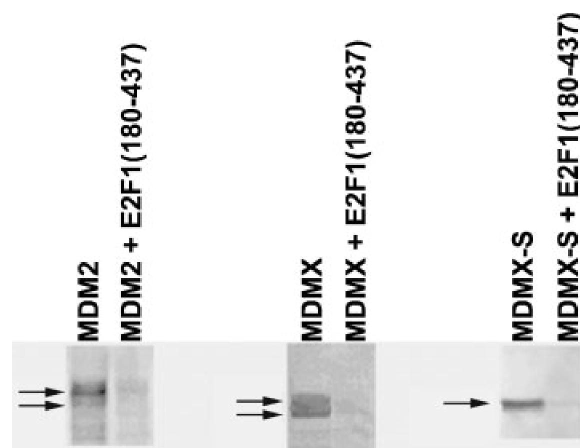

Fig. 1. E2F1 reduces MDM proteins in 293T cells. 293T cells were transiently transfected with 10 μg of expression plasmid containing MDMX, MDM2, or MDMX-S and 10 μg of either E2F1(180–437) expression plasmid or pCDNA3 control plasmid. Protein extracts were generated from transfected cells 48 h following transfection and equal amounts of extract were analyzed by immunoblot analysis. Antibodies specific for MDM2 (Ab-1, Calbiochem) or the N-terminal FLAG epitope (M2, Kodak) for MDMX or MDMX-S were used to detect the amount of protein present in the extracts. Arrows indicate the relevant MDM protein.

To determine if E2F1(180–437) could induce cell death, we transiently co-transfected 293T cells with either E2F1, E2F1(180–437), or control (pcDNA3) expression plasmid with a GFP expression plasmid. After 48 h, transfected cells were sorted based on their green fluorescence (Fig. 2A). Green fluorescent positive and negative cell fractions were re-plated individually in growth media and allowed to grow for three days. Harvested cells were fixed and analyzed for DNA content by FACS analysis. Cells within the sub-G1 population were determined to be dead. A much greater percentage of the E2F1 (12%) and E2F1(180–437) (36%) expressing 293T cells were in the sub-G1 population as compared to control transfected cells (5%) (Fig. 2B). These findings suggest that the E2F1(180–437) protein retains the ability to

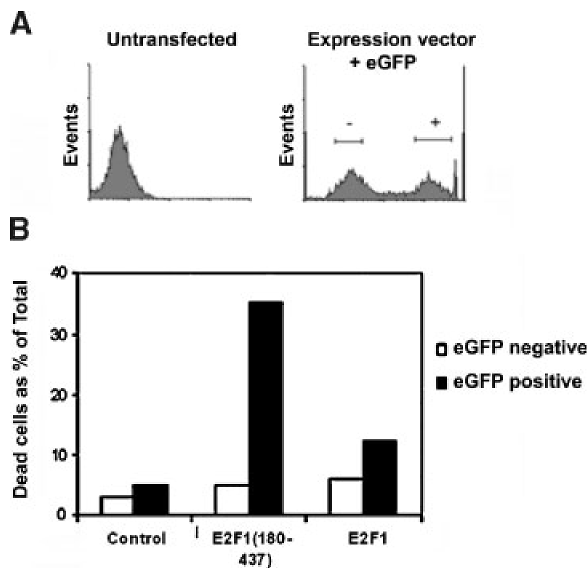

Fig. 2. E2F1(180–437) expressing cells have an increased incidence of cell death and cell cycle arrest within the G1 phase of the cell cycle. **A**: 293T cells were transiently transfected with 5 μg of Us9-EGFP expression plasmid and 15 μg of control (pcDNA3), E2F1 or E2F1(180–437) expression plasmids. A plate of cells was transfected with 20 μg of pcDNA3.1 plasmid with no Us9-EGFP was used as a negative control. Fourty eight hours after transfection, transfected cells were sorted based on green fluorescence using a Coulter EPICS Elite (Beckman Coulter, Inc.) cell sorter. Green fluorescent "positive" and "negative" cells were separated into PBS then replated in DMEM supplemented with 10% fetal calf serum for 3 days. **B**: Both Us9-EGFP positive (black bars) and negative cells (white bars) were fixed in 70% ethanol, stained with propidium iodine, and analyzed for DNA content using a Coulter EPICS XL (Beckman Coulter, Inc.) flow cytometer. The percentage of dead cells represents the number of cells in the subG1 population as a percentage of the total number of cells analyzed. Results are representative of three independent experiments.

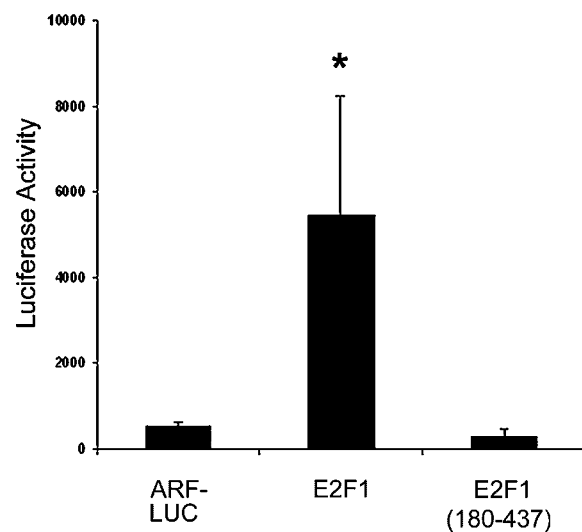

Fig. 3. The E2F1(180–437) mutant does not activate transcription. 293T cells were transfected with 5 μg of a p14/ARF luciferase reporter construct (E1β-luciferase) along with 15 μg of control (pCDNA3), E2F1 or E2F1(180–437) expression plasmids. Cells were harvested 48 h following transfection. Activation of the p14/ARF reporter was determined by measuring the luciferase activity within equal quantities of extracts from each sample. The error bars show the standard deviation. Values significantly different ($P < 0.01$) from control are indicated by an asterisk (*).

activate cell death in the absence of its DNA binding domain.

E2F1(180–437) Does Not Increase E2F1 or p53 Driven Transcription

To ensure that the activation of cell death induced by E2F1(180–437) expression was not the result of enhanced E2F1 transcriptional activity by a dominant negative or other indirect mechanism, we measured the ability of E2F1(180–437) to activate an E2F1 responsive promoter. 293T cells were transfected with either full-length E2F1, E2F1(180–437), or control pcDNA3 expression plasmid along with a luciferase reporter construct driven by the p14/ARF promoter. While the full-length E2F1 protein was able to strongly activate the p14/ARF reporter over control levels, E2F1(180–437) protein did not increase the activity from this reporter (Fig. 3). This confirms that expression of the E2F1(180–437) mutant does not result in activation of an E2F responsive promoter.

The large increase in cell death seen in E2F1(180–437) expressing cells may be the result of E2F1(180–437)-induced degradation of the anti-apoptotic MDM proteins. For example, in at least one E2F1 death pathway, a decrease in MDM proteins results in increased p53 protein levels and transcriptional activity leading to cell death [Zhu et al., 1999]. To determine whether E2F1(180–437) induced cell death was dependent on the MDM/p53 pathway, we assessed the impact of E2F1(180–437) expression on p53 stability and p53-driven transcription. To determine the effect of E2F1(180–437) on p53 stability, 293T cells were transiently co-transfected with a p53 expression plasmid and E2F1(180–437) or pcDNA3.1 as a control. p53 protein levels were then assessed by immunoblot analysis (Fig. 4A). Abundant p53 was expressed in the absence of E2F1(180–437), however, there was a decrease in p53 levels in the cells where E2F1(180–437) and p53 were co-expressed. These findings suggest that E2F1(180–437) activated cell death is

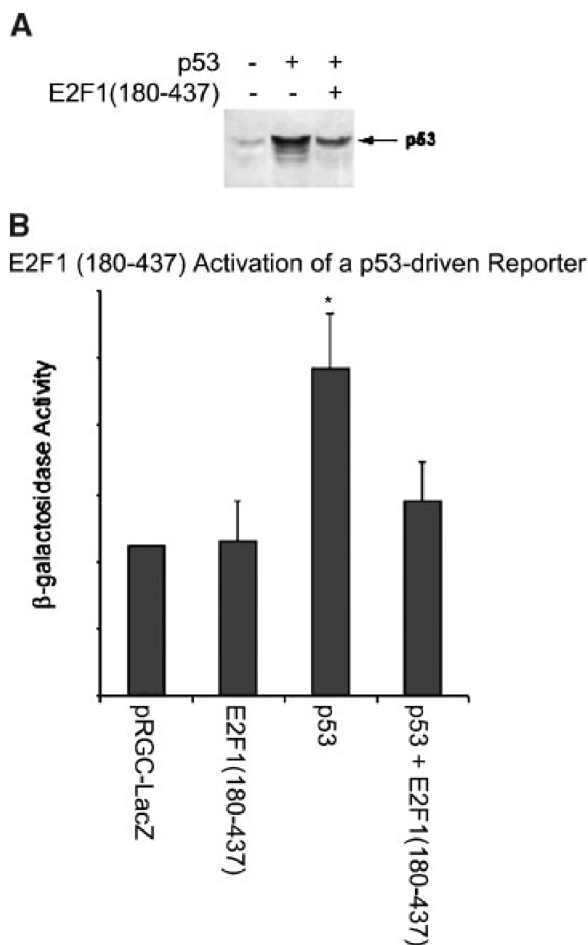

Fig. 4. The E2F1(180–437) mutant does not increase p53 protein levels or p53 driven transcription. **A**: 293T cells were transfected with 5 μg of a reporter containing several p53 sites upstream (pRGC-LacZ), in addition to 1 μg of p53 and 15 μg of E2F1(180–437) expression plasmids where indicated. Levels of p53 protein were determined by Western blot analysis using Ab-1 anti-p53 (Oncogene Sciences, Inc.) primary antibody. An arrow points to the position of p53. **B**: Activation of the pRGC-LacZ reporter was quantified by measuring the β-galactosidase activity of an equal quantity of cell extracts from control, E2F1(180–437) alone, p53 alone, or E2F1(180–437) and p53. Error bars indicate standard deviation. Values significantly different ($P < 0.01$) are indicated by an asterisk (*).

not the result of an increase in p53 protein levels and further, p53 may be a target of E2F1(180–437) induced protein degradation.

Because the MDM proteins inhibit p53 driven transcription by physically blocking the p53 transactivation domain [Oliner et al., 1993; Shvarts et al., 1996], E2F1(180–437) protein-enhanced degradation of MDM proteins may increase p53-driven transcription even while p53 levels decrease. To determine if p53 transcriptional activation was responsible for E2F1(180–437) induced death, we assessed p53-dependent transactivation from a heterologous promoter containing multiple p53 DNA binding consensus sites (pRGC-LacZ) upstream of the Lac Z reporter gene. The pRGC-LacZ reporter was co-transfected into HEK 293T cells with E2F1(180–437) alone, p53 alone or p53 and E2F1(180–437) together. As expected, the p53 protein enhanced the activity of the pRGC-LacZ reporter above that of the control (Fig. 4B). E2F1(180–437) had no effect on the pRGC-LacZ reporter alone. E2F1(180–437) did not further enhance p53-activated transcription (Fig. 4B); instead, E2F1(180–437) appears to reduce p53-activated transcription which is likely due to the reduction in p53 protein levels in cells co-expressing expressing E2F1(180–437). These findings suggest that E2F1(180–437)-activated cell death occurs through a mechanism that is p53 independent and therefore likely independent of the reduction in MDM proteins.

E2F1(180–437) Induces Calpain Activity

Our findings suggest that E2F1(180–437) induces cell death in a p53-independent manner. Since E2F1(180–437) also induces proteosome-independent degradation of MDM proteins and p53, we were interested in determining if a protease may be associated with E2F1(180–437) activated cell death. The calpains are a family of proteases distinct from caspases that are implicated in cell death [Johnson, 2000]. To determine if calpain activity was responsible for our observed changes in protein stability, we transfected 293T cells with E2F1(180–437) and stained cells for calpain-cleaved spectrin using the A38 antibody as a measurement of calpain activity [Roberts-Lewis et al., 1994]. The A38 antibody is a structural antibody specific for the 150-kDa breakdown product of spectrin cleaved by calpain. 293T cells transfected with E2F1(180–437) had a dramatic increase in levels of calpain-cleaved spectrin as detected by immunofluorescence (Fig. 5A). Transfection of full-length E2F1 did not increase calpain-cleaved spectrin over adjacent untransfected cells (Fig. 5A). E2F1(180–437) induced an increase in calpain-cleaved spectrin protein levels as determined by immunoblot analysis (Fig. 5B).

To determine whether E2F1-induced degradation of MDMX protein is dependent on calpain activation, we analyzed whether E2F1(180–

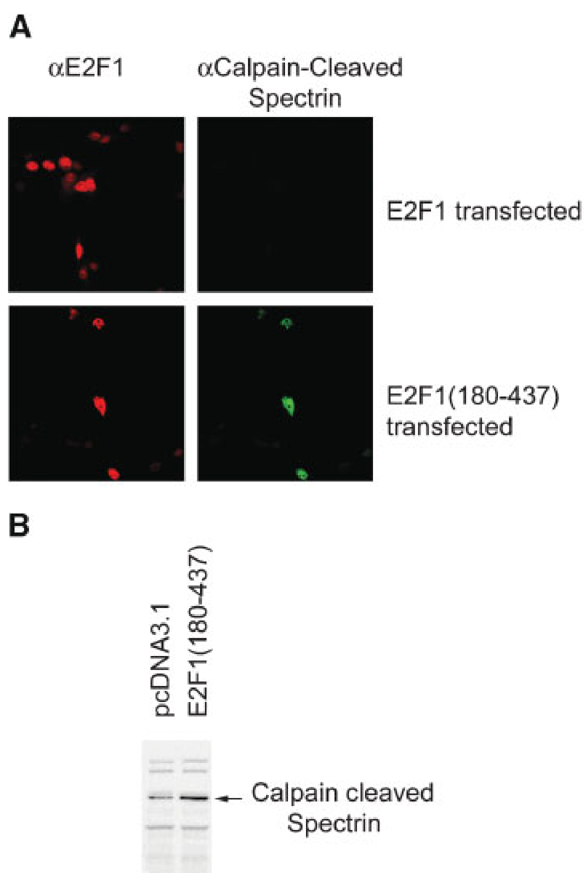

Fig. 5. E2F1(180–437) expression leads to calpain activation. **A**: 293T cells were plated on coverslips, and transfected with 20 μg of either E2F1 full length or E2F1(180–437) expression plasmids. After 48 h, cells were fixed and immunofluorescently labeled using αE2F1 (red; KH95, Santa Cruz) and α-calpain-cleaved spectrin (green; A38) primary antibodies. Using double label immunofluorescent laser confocal microscopy, we demonstrate an in increase in calpain cleaved spectrin in cell transfected with E2F1(180–437), but not E2F1. **B**: 293T cells were transfected with 20 μg of either empty pcDNA3 plasmid or E2F1(180–437) expression plasmid. 48 h post-transfection, protein extracts were made and analyzed by immunoblot analysis for calpain cleaved spectrin. Cells expressing E2F1(180–437) exhibited increased calpain-cleaved spectrin over control transfected cells.

437) expression could degrade the MDMX protein in the presence of elevated levels of the cellular calpain inhibitor, calpastatin. Co-expression of calpastatin elevated levels of the MDMX protein (Fig. 6A, calpastatin and MDMX) in both the absence and presence of E2F1(180–437) (Fig. 6A; MDMX, E2F1(180–437), and calpastatin). In addition MDMX protein is also significantly stabilized by the synthetic calpain inhibitor, calpeptin (Fig. 6B). Interestingly, the stabilization of MDMX in 293Ts by calpain inhibition even in the absence

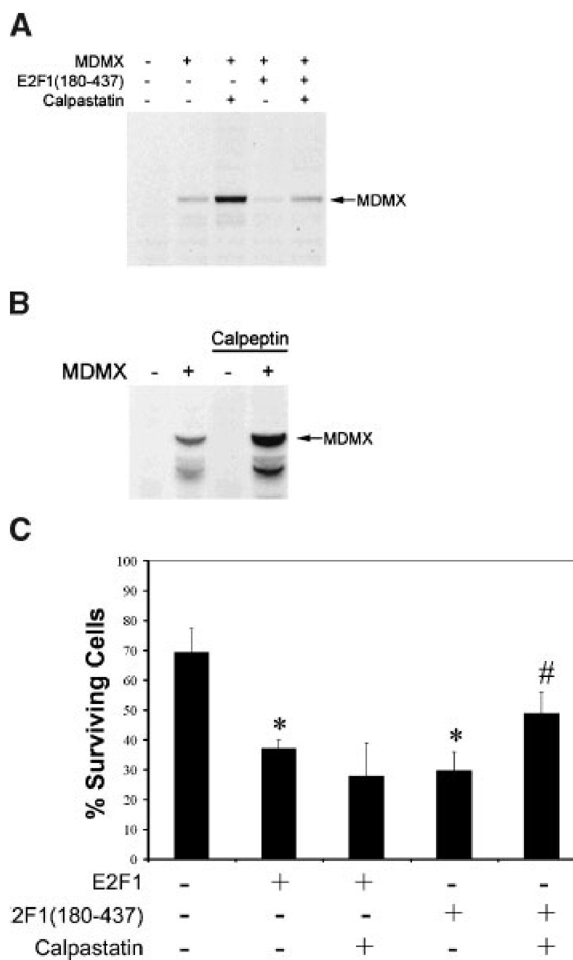

Fig. 6. Calpains mediate E2F1(180–437) induced proteolytic degradation of MDMX. **A**: 293T cells were transfected with 8 μg of MDMX, E2F1(180–437) and calpastatin expression plasmids as labeled and enough empty pCDNA3.1 expression plasmid to bring the total DNA quantity to 24 μg/transfection. After 48 h, protein extracts were made from transfected cells and equal quantities of protein were analyzed by immunoblotting for MDMX using anti-FLAG primary antibody. **B**: 293T cells were transfected with either 20 μg of MDMX expression plasmid or pCDNA3 plasmid. Twenty four hours post-transfection, either calpeptin or DMSO was added to the cells. 48 h post-transfection, protein extracts were made from transfected cells and equal quantities of protein were analyzed by immunoblotting. The MDMX protein was detected using anti-FLAG as a primary antibody. **C**: 293T cells were transfected with a total of 25 μg of total plasmid DNA consisting of 5 μg of Us9-EGFP and 10 μg of pcDNA3.1 containing either E2F1 or E2F1(180–437) and 10 μg calpastatin as labeled. 4 days after transfection, cells were stained for DNA (DAPI). Transfected GFP positive cells were counted on four coverslips and scored for the presence of a nucleus with normal morphology. Cells expressing E2F1 or E2F1(180–437) exhibited a significant reduction in the percentage of surviving transfected cells. Error bars show standard deviation. Values significantly different from control ($P < 0.01$) are indicated with an asterisk (*). Samples co-expressing calpastatin were compared to samples without calpastatin using a Student *T* test. Values significantly different ($P < 0.02$) from non-calpstatin co-expressing counterparts are indicated by an (#).

of overexpressed E2F1 supports the existence of a calpain-dependent degradation of MDMX under physiologic conditions which may or may not be dependent on E2F1. These findings suggest this protease pathway may destabilize MDMX under normal as well as pathologic stimuli.

To determine if calpain activation is mediating E2F1(180–437) induced death, we examined the ability of cellular calpain inhibitor, calpastatin, to inhibit death induced by E2F1 and E2F1(180–437). As shown previously, expression of E2F1 and E2F1(180–437) resulted in decreased cell viability (Fig. 6C). Coexpression of calpastatin provided significant protection from E2F1(180–437) induced cell death, but did not prevent E2F1 induced death in 293T cells (Fig. 6C). These findings indicate that E2F1(180–437) induced death is partially dependent on calpain activation.

MDMX and E2F1 Protein Levels are Inversely Proportional in SIV and SIVE Neurons

Increased cytoplasmic E2F1 has been observed in neurons of both HIV encephalitis (HIVE) and the simian form of the disease, SIV encephalitis (SIVE) [Jordan-Sciutto et al., 2000a,b, 2002a,b]. To determine if cytoplasmic E2F1 may indicate a change in MDMX stability, we immunostained brain sections from SIV encephalitic, SIV non-encephalitic, and uninfected, non-encephalitic control macaques for E2F1 and MDMX. Using triple label immunofluorescent laser confocal microscopy, E2F1 immunofluorescence, using the KH95 antibody, is strikingly higher in the cytoplasm of neurons from SIVE animals as compared to SIV and uninfected controls as previously described (Fig. 7; data not shown [Jordan-Sciutto et al., 2000a,b]). The MDMX protein is readily detectable in the cytoplasm of neurons in control and SIV animals (Fig. 7, SIV—left panel, data not shown), staining for MDMX protein is reduced dramatically in the neurons of macaques with SIVE (Fig. 7, right panel). We observed these same results in caudate and mid frontal cortex of three uninfected control, five SIV, and four SIVE animals.

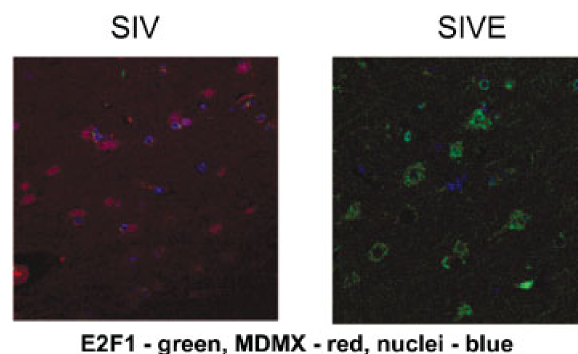

Fig. 7. An elevation in E2F1 protein levels in SIVE neurons are associated with a decrease in MDMX protein. Using triple label immunofluorescent confocal microscopy for E2F1 (green), MDMX (red), and nuclei (blue), we assessed staining for these three proteins in midfrontal cortex and basal ganglia of three uninfected, non-encephalitic macaques (data not shown), five SIV infected, non-encephalitic macaques (SIV), and four SIV-infected animals with encephalitis (SIVE). In mid frontal cortex of control and SIV cases, we observed MDMX staining (red) in a majority of cells with large nuclei which are most likely neurons (SIV). We did not observe much E2F1 staining (green) in control or SIV cases as previously reported (**left panel**). In SIVE cases (**right panel**), we observed minimal MDMX staining (red) in neurons exhibiting increased expression of E2F1 (green). Bar = 20 μM.

DISCUSSION

Activation of cell death has been attributed to several pathways largely involving direct transcriptional activation of genes such as p14/ARF, p73, and Apaf-1 [Ginsberg, 2002]. Here we report a novel method of E2F1-induced cell death which does not require DNA binding activity or p53 stabilization. Using an E2F1 mutant lacking the DNA binding domain and nuclear localization sequence, E2F1(180–437), we still observe cell death of HEK293T cells in the absence of transcriptional activation. E2F1(180–437)-induced cell death is associated with an increase in calpain activity as detected by increased calpain-cleaved spectrin, as well as increased proteolytic degradation of several cytoplasmic proteins by cathepsin proteases which are normally confined to the lysosome [Strachan et al., 2001]. Taken together our results suggest that elevated E2F1 protein levels augment a calpain-cathepsin cell death pathway [Yamashima, 2000]. A down stream target of E2F1 activation of this protease cascade is decreased stability of MDMX. Consistent with our in vitro observations, we observe a significant reduction in MDMX protein in neurons of SIVE macaques expressing increased levels of E2F1 as compared to SIV. The reciprocal localization of MDMX and E2F1 in neurons of encephalitic macaques provides a scenario in which this cell death pathway may occur in vivo.

A Novel Mechanism of E2F1 Induced Apoptosis

Among E2F family members, E2F1 alone has been implicated in inducing apoptosis [DeGregori et al., 1997]. Because the well-characterized E2F1-induced apoptotic pathways involve transcriptional activation, it is not clear why only E2F1 induces apoptosis and not it's closely related family members, E2F2 and E2F3. By creating chimeric proteins consisting of homologous regions of E2F1 and E2F3, Hallstrom and Nevins [Hallstrom and Nevins, 2003] have defined the domain responsible for E2F1-specific induction of apoptosis. These experiments identified the domain contained between the DP1 dimerization domain and the acidic activation domain as responsible for activation of E2F1-specific apoptosis. Interestingly, our E2F1 mutant also contains this "apoptosis" inducing region. Future experiments will determine if this motif is responsible for the activation of calpains observed in our experimental models and how cytoplasmic E2F1 contributes to calpain activation.

Elevated E2F1 protein, increased μ-calpain activity, and activation of cell cycle checkpoints have been associated with the cellular response to the compound β-lapachone. β-lapachone is currently being explored as a novel cancer therapeutic for its ability to selectively elevate the E2F1 protein in the absence of DNA damage [Li et al., 2003]. Increased E2F1 protein results in the activation of a cell cycle checkpoint, which leads to the selective killing of cancerous cells. Interestingly, β-lapachone has also been shown to activate μ-calpain during its induction of cell death [Tagliarino et al., 2001, 2003], further suggesting the existence of a link between E2F-1 and calpain in cell death induction.

Implications for E2F1 Induction of Calpains and Cathepsins

Despite extensive investigation, the mechanism of neuronal loss in neurodegenerative diseases is still controversial. The ability of E2F1 to activate calpains may account for the mixture of apoptotic and necrotic markers observed in neurodegenerative diseases. Prolonged activation of calpains has been shown to cause loss of lysosomal membrane integrity resulting in lysosomal proteases leaking into the cytoplasm and the induction of cell death [Yamashima et al., 2003]. The calpain-cathepsin cell death cascade is believed to contribute to both apoptotic and necrotic cell death (Fig. 8). While moderate rupture of lysosomal membranes leads

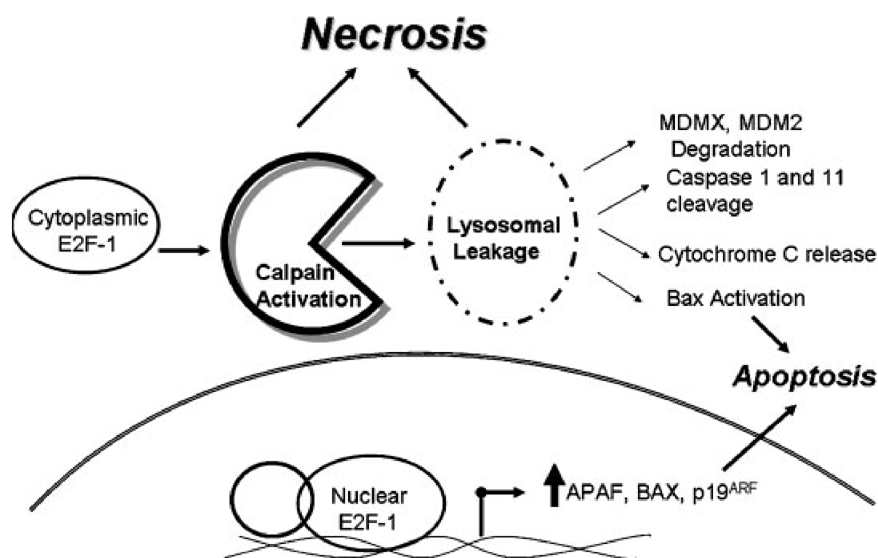

Fig. 8. Model of E2F1 induced cell death. E2F1 has been shown to play a role in inducing apoptotic cell death by binding DNA and activating transcription of BAX, APAF, and p19^{ARF}. This mode of death requires E2F1 to bind DNA in the nucleus and activate transcription. Our findings indicate that E2F1 can activate an alternative death pathway independent of DNA binding, transcriptional activity, and a nuclear localization signal. In this pathway, E2F1 induces calpain activation which can induce necrosis or lysosomal leakage. Lysosomal leakage leads to degradation of MDM proteins, Bax activation, caspase 1 and 11 activation, and cytochrome C release. Depending on the degree of lysosomal leakage the cell may undergo apoptosis via Bax activation and cytochrome C release or necrosis.

to conventional caspase activation and apoptosis through downstream degradation of anti-apoptotic and upregulation of pro-apotpotic signals, more severe leakage has been suggested to kill cells by necrosis without caspase activation [Brunk et al., 1997; Kagedal et al., 2001]. Genetic screens for inhibitors of necrosis have identified several intracellular Ca^{2+} binding proteins highlighting the importance of calpain activation [Xu et al., 2001]. We have previously reported that the over-expression of E2F1(180–437) in phoenix cells activates the proteolytic degradation of MDMX, MDMX-S, and MDM2 [Strachan et al., 2001]. When bafilomycin A1 was used to inhibit pH dependent lysosomal proteases, we demonstrated that E2F1(180–437) degradation of MDM proteins were likely due to cathepsin proteases leaking out of the lysosome. In our current model, E2F1(180–437) over-expression does not initiate a classic E2F1-induced apoptotic response by increasing stability of p53 resulting from destabilization of MDM proteins as would be predicted from previous findings. Instead, E2F1 over-expression leads to activation of calpain and cathepsins in the absence of p53 stabilization and transcriptional activity. If MDMX is participating in this death pathway, our results suggest its role is distinct from its known function in regulating p53 stability. Further investigation is needed to elucidate the role of MDMX in this new mode of E2F1, calpain dependent cell death.

In addition to inducing apoptosis through degradation of MDM proteins, moderate lysosomal leakage has been reported to induce apoptosis by several other mechanisms. Lysosomal leakage has been reported to induce direct cleavage of procaspase 1 and 11 by cathepsins [Schotte et al., 1998; Vancompernolle et al., 1998]. Leakage of cathepsin B has also been shown to promote the release of cytocrome c from the mitochondria [Guicciardi et al., 2000], resulting from lysosomal proteases cleaving and, therefore, activating the pro-apoptotic, Bcl-2 family member, Bax [Bidere et al., 2003]. Lysosomal leakage provides another mechanism by which E2F1 controls cellular viability (Fig. 8).

The E2F1-Calpain-Cathepsin Pathway in Neuronal Cell Loss

In addition to the requirement for E2F1 in the activation of neuronal cell death in several in vitro models of neurodegeneration, the calpain-cathepsin cell death pathway is strongly associated with aspects of neurodegeneration. Genetic lysosomal protease disorders lead to impaired brain development and share similar pathologies to those of neurodegenerative diseases [Bahr and Bendiske, 2002]. In an in vitro model of Parkinson's disease, oxidative damage by 6-hydroxydopamine can induce release of proteases from the lysosome [Takai et al., 1998]. Similarly, endosomal accumulation of the Aβ peptide, a model of Alzheimer's disease, has been shown to contribute to lysosomal leakage [Yang et al., 1998]. Finally, normal aging, a common predisposing factor for neurodegenerative diseases, lowers cathepsin L and cathepsin B activity [Amano et al., 1995], while increasing cathepsin D and cathepsin E [Nakanishi et al., 1997] suggesting that changes in lysosomal activity may account for the contribution of aging to disease development.

In addition to inducing lysosomal leakage, calpains have been reported to target cleavage of several proteins implicated in progression of neurodegenerative disease including the mutant huntingtin protein [Kim et al., 2001] and p35 [Lee et al., 2000], the activating subunit of cdk5. Finally, inhibition of calpains has been shown to prevent dopaminergic neuron loss in an animal model of Parkinson's disease [Crocker et al., 2003].

Our observations that E2F1 induces calpain activation suggest E2F1 may initiate events associated with pathogenesis of neurodegenerative diseases. In support of this, E2F1 has been shown to exhibit increased immunostaining in multiple neurodegenerative diseases including HIVE, SIVE, Alzheimer's disease, and amyotrophic lateral sclerosis [Jordan-Sciutto et al., 2000a,b, 2001, 2002a,b; Ranganathan and Bowser, 2003]. Interestingly, in these studies, E2F1 is predominantly cytoplasmic in neurons. Initially, we interpreted this localization to indicate that E2F1 is being sequestered from the nucleus to prevent activation of classic apoptosis. Our present findings indicate that instead, E2F1 may have a novel role in the cytoplasm regulating calpain activity. This is interesting given the growing number of transcription factors and kinases exhibiting apparently "aberrant" or altered localization in neurodegenerative diseases. Perhaps these proteins which include

myc associated zinc finger protein (MAZi/ZF87), fetal alz-50 clone 1 (FAC1), activating transcription factor-2 (ATF2), cyclin dependent kinase 4, c-jun N terminal kinase (JNK), cyclin A, and extracellular signal regulated kinase (ERK) [Schoonover et al., 1996; McShea et al., 1997; Yamada et al., 1997; Tsujioka et al., 1999; Jordan-Sciutto et al., 2000a,b; Kulich and Chu, 2001; Zhu et al., 2001; Jordan-Sciutto et al., 2002a,b; Ranganathan and Bowser, 2003; Yang et al., 2003], are not aberrantly localized, but are also performing functions that have not yet been ascribed to these proteins. Taken together, these findings indicate that E2F1, calpain-dependent death may be the one of several novel pathways regulating neuronal loss in neurodegenerative disease. Understanding the mechanism by which E2F1 induces calpains has the potential to elucidate an important pathway regulating neuronal viability and provide novel targets for therapy.

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