Unbound E2F modulates TGF-β1-induced apoptosis in HuH-7 cells

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Summary
E2F is an important target of the retinoblastoma protein (pRb) and plays a critical role in G1/S progression through the cell cycle. TGF-β1 arrests HuH-7 cells in G1 by suppressing phosphorylation of pRb and induces apoptosis by inhibiting its expression. In this study, we examined the downstream effects of TGF-β1-induced apoptosis and the potential roles for pRb and E2F. The results indicated that greater than 90% of the TGF-β1-induced preapoptotic cells were arrested in G1 phase of the cell cycle. This was associated with a significant increase in both E2F-DNA-binding activity and transcription of E2F-responsive reporter constructs. In contrast, no significant changes were observed in E2F mRNA and protein levels, and the overexpression of pRb partially inhibited E2F activation. Gel-shift assays identified more than four E2F complexes from preapoptotic and synchronized G1 HuH-7 cells, each exhibiting different patterns of E2F-associated proteins. The increased E2F activity did not affect the association patterns with pRb, p107 and p130, but altered the formation of an E2F–DP-1 complex. In contrast, E2F–DP-2 exhibited little change in the preapoptotic cells. Moreover, TGF-β1-induced apoptosis at G1 and inhibited entry into S phase irrespective of the increased E2F activity. The release of preapoptotic cells from TGF-β1 resulted in rapid S phase entry and subsequent apoptosis in 33% of cells over a 72 hour period. In conclusion, the results demonstrate that TGF-β1-induced apoptosis in HuH-7 cells is associated with a marked increase in activity of transcription factor E2F that is partially inhibited by overexpression of pRb. Preapoptotic changes are, in part, reversible upon removal of TGF-β1 and the majority of cells re-enter the normal cell cycle. Finally, TGF-β1-induced apoptosis with the associated increase in E2F activity can occur in both the G1 and S phases of the cell cycle.

Key words: Apoptosis, Cytokine, E2F transcription factors, Human hepatoma cells, TGF-β1

Introduction
The E2F transcription factors have been shown to regulate expression of a panel of cellular genes that control DNA synthesis and proliferation, and appear to be dependent on the cell cycle. The retinoblastoma protein (pRb) together with other cellular factors regulates the activity of the E2F family of proteins (Dyson, 1998). The transcriptional activity of E2F was originally identified by its DNA-binding activity to a specific recognition sequence in the adenovirus E2 promoter (Harbour and Dean, 2000). Characterization of the DNA-binding complex revealed that it was a heterodimer containing an E2F family member and a DP transcription factor (Bagchi et al., 1990; Hiebert et al., 1995). While E2F can bind to DNA in vitro, DP proteins bind to DNA with low affinity (Girling et al., 1993). Dimerization of DP proteins with E2F proteins increases the transcription activity of E2F and is required for association of E2F with pRb (Helin et al., 1993; Krek et al., 1993; Beijersbergen et al., 1994; Sardet et al., 1995). To date, five highly related E2F genes (E2F-1 to -5) and three DP genes have been identified in vertebrates (Dyson, 1998). E2F-1, -2, and -3 can associate with a DP member and then bind to pRb through a small 18 amino acid interaction domain located in the C-termini of the E2F polypeptides (Kaelin et al., 1992; Lees et al., 1993). The remaining two E2Fs, E2F-4 and E2F-5, also associate with a DP member, but bind to the pRb-related proteins, p107 and p130 (Beijersbergen et al., 1994; Hijmans et al., 1995; Sardet et al., 1995).

It has been reported that overexpression of E2F-1 induces cells to undergo apoptosis, and can occur by both p53-dependent and -independent mechanisms (Holmberg et al., 1998). The cooperation between E2F-1 and p53 may provide an apoptotic signal when it occurs simultaneously with an arrest of cell cycle progression, such as by p53. The ability of E2F-1 to induce apoptosis was thought to be a unique feature compared with the other members of the E2F transcription family (DeGregori et al., 1997). However, it has subsequently been determined that other members of the E2F family (Harbour and Dean, 2000), in particular E2F-3 (Ziebold et al., 2001), make major contributions to the apoptotic pathway. In fact, it has recently been reported that Apaf-1, the gene for apoptosis protease-activating factor 1, is a transcriptional target for both E2F and p53 (Moroni et al., 2001).

TGF-β1 is a potent growth inhibitor, and can induce rapid growth arrest and apoptosis in many cell types, including hepatic cells in culture and in vivo (Oberhammer et al., 1992; Fan et al., 1995). Its growth suppressive effects appear to be linked, in part, to decreased phosphorylation of pRb. Also, the inhibition of pRb expression by TGF-β1 in human HuH-7 hepatoma cells is associated with significant apoptosis. The
inactivation of pRb either by phosphorylation, mutation, or binding to an oncprotein results in the loss of its ability to sequester E2F-1 as a pRb–E2F-1 complex, and an increase in unbound E2F-1 (Riley et al., 1994; Fan and Steer, 1996). E2F-1 is the best-characterized member of the E2F transcription factor family (Kaelin et al., 1992; Shan et al., 1996), and its overexpression is sufficient to offset pRb-mediated G1 arrest and thereby promote S phase entry (Johnson et al., 1994). This is presumed to occur through transactivation of the E2F-1 target genes dihydrofolate reductase (DHFR) (Blake and Azizkhan, 1989), thymidylate synthase, and other factors involved in entry and progression through S phase (Ishida et al., 2001; Müller et al., 2001).

It is now well established that apoptosis is a gene-directed process and involves an intrinsic, albeit complex, cell death program that is regulated by the cell cycle (Kroemer et al., 1995). In fact, cell cycle progression and programmed cell death appear to share a number of common pathways, as well as specific factors (Evan et al., 1995). For example, pRb is an important cell cycle regulator and functions to inhibit cell proliferation by complexing with transcription factor E2F at the G1/S check-point. However, it is also known that the loss of functional pRb can induce apoptosis while its expression can preserve cell survival (Haas-Kogan et al., 1995; Fan and Steer, 1996). Interestingly, TGF-β1-induced apoptosis was preceded by cell cycle arrest in HuH-7 cells. In fact, 93% of the preapoptotic cells were initially arrested in G1 and eventually died from apoptosis with continued exposure to TGF-β1 (Fan et al., 1996). In contrast, deregulated expression of E2F-1 can drive quiescent cells into S phase, ultimately resulting in apoptosis (Qin et al., 1994; Shan and Lee, 1994). The mechanism by which TGF-β1 induces apoptosis through deregulation of pRb expression and phosphorylation in HuH-7 cells may, in fact, involve a number of factors, although E2F-1 appears to be a key regulator in this pathway. In fact, the abundance and accessibility of E2F-1 is critical to the regulation of the cell cycle by pRb. Untimely activation of E2F-1 and/or its overexpression may act to initiate TGF-β1-induced apoptosis.

In this study, we report that E2F-DNA-binding activity is significantly increased in TGF-β1-induced apoptosis of HuH-7 cells, in part, through the loss of interaction with pRb. The increased levels of unbound E2F family members may result in transactivation of several well-characterized cell-cycle-regulated genes. While the role of E2F in apoptosis is closely linked to its interaction with pRb, it functions in a complex array of pathways that occur in both the G1 and S phases of the cell cycle. The preapoptotic changes are partially reversible only upon removal of TGF-β1, and can be inhibited by overexpression of pRb. The E2F family members are key regulators of cell survival and cell death.

**Materials and Methods**

**Cell culture and synchronization**

HuH-7 cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) (Atlanta Biologicals, Norcross, GA) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin (Life Technologies, Gaithersburg, MD) and 10% fetal calf serum (FBS) (Atlanta Biologicals). The cells were incubated at 37°C±1 mM TGF-β1 (R and D Systems, Minneapolis, MN) for 72 hours as described previously (Fan and Steer, 1996). The cultures were then gently washed three times with 1× phosphate buffered saline (PBS), pH 7.4, to remove nonadherent dead cells. The adherent viable cells were then either released from TGF-β1 with fresh media every 24 hours, or harvested for flow cytometry or preparation of nuclear extracts. The released cells were harvested after 15, 24, 48 and 72 hours and analyzed by flow cytometry.

For G1 phase synchronization, cells were starved in DMEM medium containing 0.1% FBS for 48 hours, then replaced with isoleucine free medium (Atlanta Biologicals) containing 5 μM each of deoxycytidine, deoxyadenosine, deoxyguanosine and deoxythymidine (Sigma, St Louis, MO) for 30 hours. The efficiency of synchronization was monitored by flow cytometry. For G1/S synchronization, the medium was removed after G1 phase synchronization and the cells were washed twice with 1× PBS, pH 7.4. The cells were incubated with fresh DMEM containing 10% FBS plus aphidicolin (2.5 μg/ml) for 18 hours, and then washed twice with 1× PBS. For S phase synchronization, fresh DMEM medium supplemented with 10% FBS was added and the cells incubated for an additional 2 hours before harvesting. For G2/M phase, the cells were incubated in DMEM supplemented with 10% FBS and 80 ng/ml of nocodazole for 18 hours, with the addition of 0.06 μg/ml colcemid for the final 2 hours prior to harvesting.

**Flow cytometry**

HuH-7 cells were harvested after either TGF-β1 incubation or synchronization, and analyzed by flow cytometry as described previously (Fan et al., 1996). In brief, the culture medium was collected and combined with the PBS washes. The attached cells were removed with trypsin and were centrifuged at 500 g for 10 minutes at 4°C. The resulting pellets (~5×10⁶ cells) were resuspended in 0.5 ml of ice-cold 70% ethanol and maintained on ice for at least 30 minutes. The cells were then centrifuged and resuspended in 0.5 ml of PBS containing 0.1 mg/ml RNase A (Roche Diagnostics, Indianapolis, IN), 50 μg/ml propidium iodide (Sigma), and 0.05% (v/v) Triton X-100 (Sigma), and incubated for 45 minutes at room temperature. The samples were analyzed using a FACS Star Plus sorter (Becton-Dickinson, San Jose, CA) with 200 mW argon laser excitation at 488 nm. Cell clumps were identified and gated out by plotting integral red fluorescence versus peak height. The cell cycle distribution was established by plotting the intensity of the propidium iodine signal, which reflects the cellular DNA content. Apoptotic cells were identified as a hypodiploid DNA peak representing cells that contained less than a 2N DNA content.

**Preparation of nuclear extracts**

All reagents were from Sigma unless otherwise indicated. The cells were lysed with hypotonic buffer (10 mM Heps pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 0.5 mM NaF, 0.5 mM Na₃VO₄) on ice for 20 minutes and dounce homogenized using ten strokes with the B pestle. The lysate was centrifuged at 3300 g for 15 minutes at 4°C, the supernatant discarded and the nuclear pellet resuspended in 1.66 volumes of extraction buffer (20 mM Heps, pH 7.6, 1.6 M KCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM NaF, 0.5 mM Na₃VO₄, 0.5 mM PMSF, 1 mM DTT, 1 mg/ml leupeptin and aprozin) and gently stirred at 4°C for 30 minutes. The sample was centrifuged at 32,000 g for 1 hour at 4°C, and the supernatant dialyzed for 1 hour at 4°C against 20 mM Heps, 0.1 mM EDTA, 0.5 mM NaF, 0.5 mM Na₃VO₄, 0.5 mM PMSF, 1 mM DTT, 1 mg/ml leupeptin and aprozin. Protein concentrations of the dialyzed nuclear extracts were determined by the Bradford method using a commercial protein assay system (Bio-Rad Laboratories, Hercules, CA). The extracts were then aliquoted, flash-frozen in liquid nitrogen and stored at −70°C.
E2F mediates apoptosis by TGF-β1 in HuH-7 cells

Gel-shift assays
E2F electrophoretic mobility shift assays were performed as described previously (Cao et al., 1992). The E2F-specific (GAT TTA AGT TTC GCG CCC TTT CTC AA) or mutant (GAT TTA AGT TTC GAT CCC TTT CTC AA) synthetic oligonucleotides (Integrated DNA Technologies, Coralville, IA) were labeled with [α-32P]dCTP. Mobility shift assays were performed in 20 μl reaction mixtures containing 20 μg of nuclear extract in 20 mM Hepes, pH 7.6 with 40 mM KCl, 1 mM MgCl2, 0.1 mM EGTA, 0.4 mM DTT, 5 μg BSA, 2.5% (v/v) Ficoll, 2 μg of salmon sperm DNA and 32P-labeled probe at 20,000-50,000 cpm. ‘Supershift’ experiments were performed by incubating extracts with 2 μg of the relevant antibodies for 10 minutes on ice. These included anti-pRb XZ161 generously provided by Ed Harlow (Harvard Medical School, Boston, MA), anti-p107, anti-p130, anti-DP-1, anti-DP-2, anti-E2F-1, anti-E2F-2, anti-E2F-3 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-MDM2 (Oncogene Research Products, Boston, MA). For binding specificity, 10-, 100- and 1000-fold excess of unlabeled E2F-specific oligonucleotides were coincubated with the 32P-labeled probes. To disrupt protein-protein interactions within the E2F complexes, nuclear extracts were incubated with increasing concentrations of sodium deoxycholate (DOC) (0.2%, 0.4%, 0.8%, 1.2%; v/v) for 20 minutes on ice and then NP-40 was added to a final concentration of 0.7% (Bagchi et al., 1990). The mobility shift assay reactions were incubated at room temperature for 30 minutes, the samples were then loaded on a 0.25% Tris-Borate-EDTA, 4% polyacrylamide gel (30:1) and electrophoresed at 200 V for 2 hours. Gels were dried and analyzed by autoradiography. The relative intensities of the bands were determined using a BioRad model GS-700 imaging densitometer (Bio-Rad Laboratories).

Transfections and CAT assays
Transfections were performed as previously described (Fan et al., 1996). In brief, 5 μg of each construct was transfected into HuH-7 cells for 24 hours using Lipofectin™ (Life Technologies) according to the manufacturer’s recommendations. The reporter construct E2F1CAT consisted of the entire human E2F-1 promoter fused to the chloramphenicol acetyltransferase (CAT) gene (Johnson et al., 1993); 4XE2FCAT was constructed with a synthetic promoter containing four E2F consensus binding sites (Ohtani and Nievins, 1994) driving CAT expression. Cells were co-transfected with a pRb expression construct pCMV Rb containing a CMV promoter (Fan et al., 1996). A lacZ β-galactosidase reporter construct was used as a control plasmid. The cells were then incubated for an additional 36 hours with or without 1 nM TGF-β1. After removing dead cells by gentle washing, the remaining viable cells were harvested for preparation of cell lysates for CAT ELISA assays (Roche Diagnostics), which were performed according to the manufacturer’s specifications. All CAT activity was normalized to the observed β-galactosidase expression, thus controlling for differences in transfection efficiency and nonspecific effects of cell culture.

Northern and western blot analysis
Total RNA was prepared from the cells, electrophoresed and transferred to MSI MagnaGraph nylon membranes (Micron Separations, Westboro, MA) as previously described (Johnson et al., 1993; Fan et al., 1996). Lane loading was determined by ethidium bromide staining and densitometric analysis as described previously (Correa-Rotter et al., 1992). Northern blots were probed with a 0.7 kb EcoRI/BglII E2F-1 cDNA fragment that was labeled and detected using the DIG/Genius™ System (Roche Diagnostics) according to the manufacturer’s protocol. Whole cell lysates were prepared and analyzed by western blotting as described previously (Fan et al., 1996) using monoclonal anti-E2F-1 antibody (Santa Cruz Biotechnology).

**Fig. 1.** Morphological and cell cycle analyses of synchronized G1 and TGF-β1-induced apoptotic HuH-7 cells. The cells were treated with 1 nM TGF-β1 for 72 hours (left panels) or synchronized at G1 (right panels) as described in Materials and Methods. (A) Fluorescent labeling of nuclei with Hoechst 333258 showing the characteristic nuclear fragmentation associated with TGF-β1-induced apoptosis (left). (B) Analysis of cell cycle phase by flow cytometry after removing apoptotic cells. The percentage of total cells present in the different cell cycle phases is indicated in the top-right panel.

Immunohistochemistry
The cells were fixed with 4% paraformaldehyde (w/v) in 1× PBS, pH 7.4 at room temperature for 10 minutes, washed three times with 1× PBS, and then stained with Hoechst dye 33258 (1 μg/ml) for 5 minutes and mounted using an anti-fade reagent. The images were acquired and the nuclear morphology was analyzed as described previously (Fan et al., 1996).

Results
E2F activity is increased in TGF-β1-induced preapoptotic G1 cells
It is well established that the phosphorylation state of pRb regulates E2F-1 activity by modulating levels of unbound E2F-1. Our previous studies on TGF-β1-induced apoptosis revealed that it inhibits both the expression and phosphorylation of pRb (Fan et al., 1996), suggesting that E2F-1 could be an important factor in modulating this process. Therefore, HuH-7 cells were incubated with 1 nM recombinant human TGF-β1 for 72 hours and exhibited a significant level of apoptosis by Hoechst staining (Fig. 1A, left panel). After removal of the apoptotic cells, flow cytometry analysis of the preapoptotic cells indicated that more than 91% of the cells were in G1 phase (Fig. 1B, left panel). A similar distribution was observed for normal HuH-7 cells synchronized in the G1 cell cycle (Fig. 1B, right panel).

Nuclear extracts were prepared from synchronized G1-, S- and G2/M phase HuH-7 cells or TGF-β1-induced preapoptotic cells. Gel retardation assays of E2F–DNA-binding complexes indicated at least six different components in synchronized and preapoptotic G1 nuclear extracts (Fig. 2). The same four E2F–DNA-binding complexes were present in all samples prepared with the extracts from the normal synchronized cells,
The position and numerical designation of the 32 P-labeled probe containing no nuclear extracts were devoid of activity (lanes 13, 14). Excess of unlabeled mutant E2F oligonucleotide (lanes 8, 12) during 6, 10) and 1000-fold (lanes 7, 11) molar excess, or a 1000-fold molar excess of unlabeled wild-type E2F oligonucleotides were added at 10-fold (lanes 5, 9), 100-fold (lanes 6, 10) and 1000-fold (lanes 7, 11) molar excess, or a 1000-fold molar excess of unlabeled mutant E2F oligonucleotide (lanes 8, 12) during the 30 minute incubation for binding specificity. Negative controls containing no nuclear extracts were devoid of activity (lanes 13, 14). The position and numerical designation of the 32 P-labeled DNA–protein complexes are indicated on the left. The cell-cycle-associated phases of the nuclear extracts are indicated at the top, mt, mutant E2F oligonucleotide; TGF-β1, preapoptotic nuclear extract from HuH-7 cells incubated with TGF-β1 for 72 hours; wt, wild-type E2F oligonucleotide.

while two novel complexes (I and IV) were observed using extracts from the preapoptotic cells (Fig. 2, lanes 1-4). Most of the E2F-binding activity from the normal G1 cells was detected in complexes II and III (Fig. 2, lane 1). In contrast, the majority of increased E2F activity in the TGF-β1-induced preapoptotic G1 cells was associated with complexes I, IV, V and VI (Fig. 2, lane 2). The total E2F-binding activity in S phase (measured as Σ band intensities) was greater than either the G1- or G2/M phases of the synchronized normal cells (Fig. 2, lanes 1, 3, 4). However, much higher E2F activity was observed in TGF-β1-induced G1 preapoptotic cells than in the synchronized G1-, S- and G2/M-phase normal cells (Fig. 2, lanes 1-4). Interestingly, dramatic increases in E2F complexes V and VI relative to G1 phase were also detected in reactions using S phase nuclear extracts.

To determine the specificity of E2F binding, incubations were done with 10-, 100-, and 1000-fold excess of unlabeled wild-type or mutant E2F oligonucleotides. The results indicated that a 1000-fold excess of unlabeled wild-type oligonucleotide could effectively compete for the E2F-binding activity in either of the G1 nuclear extracts. In contrast, the mutant E2F oligonucleotide showed no effect (Fig. 2, lanes 5-12). Interestingly, the wild-type E2F oligonucleotides resulted in the rapid loss of complexes I to IV in both G1 synchronized populations, while the 1000-fold excess was required to diminish complexes V and VI.

Fig. 2. E2F mobility-shift assays of nuclear extracts from synchronized G1 and TGF-β1-induced preapoptotic cells. Nuclear extracts were prepared, incubated with wild-type 32P-labeled E2F oligonucleotide probe and analyzed by PAGE (lanes 1-12) as described in Materials and Methods. Unlabeled wild-type E2F oligonucleotides were added at 10-fold (lanes 5, 9), 100-fold (lanes 6, 10) and 1000-fold (lanes 7, 11) molar excess, or a 1000-fold molar excess of unlabeled mutant E2F oligonucleotide (lanes 8, 12) during the 30 minute incubation for binding specificity. Negative controls containing no nuclear extracts were devoid of activity (lanes 13, 14). The position and numerical designation of the 32 P-labeled DNA–protein complexes are indicated on the left. The cell cycle-associated phases of the nuclear extracts are indicated at the top, mt, mutant E2F oligonucleotide; TGF-β1, preapoptotic nuclear extract from HuH-7 cells incubated with TGF-β1 for 72 hours; wt, wild-type E2F oligonucleotide.

Increased E2F activity results from unbound E2F and not changes in transcript or protein expression

We determined whether the increased E2F-DNA binding activity observed in both the synchronized S phase and TGF-β1-treated preapoptotic cells resulted from increased E2F-1 mRNA and/or protein steady-state levels. Both northern and western blot analyses were performed on the same samples. In fact, the results indicated no significant changes in the steady-state levels of either the E2F-1 transcript or protein during the cell cycle (Fig. 3A, B). Although the expression of E2F-1 mRNA was minimally increased in TGF-β1-induced preapoptotic G1 cells (less than twofold), no significant change in protein abundance was observed (Fig. 3A, B). However, we could not rule out changes in rates of de novo synthesis versus degradation of E2F protein and/or transcript in the different cell cycle phases.

E2F–protein complexes are different in the two G1 cell populations

We detected at least six different E2F-DNA complexes in the nuclear extracts of both synchronized and TGF-β1-induced preapoptotic G1 cells, with perhaps only two in common (Fig. 4, lanes 1, 6). These results suggested that E2F cooperates with different cellular proteins in G1 preapoptotic cells, permitting interactions with cell cycle mediators distinct from those in synchronized G1 cells. To further characterize these E2F–protein complexes, we performed sodium deoxycholate (DOC) dissociation assays (Baeuerle and Baltimore, 1988). DOC-induced loss of specific DNA–protein complexes results from disruption of protein-protein interactions in complexes containing at least two protein species (Bagchi et al., 1990). Thus, nuclear extracts from both synchronized and preapoptotic G1 cells were incubated with DOC, resulting in a concentration-dependent dissociation of complexes I to VI. While complexes I to IV were completely dissociated with 0.8% DOC (Fig. 4, lanes 4, 9), complex V was more sensitive and dissociated at 0.4% DOC. In contrast, complex VI was
relatively resistant to DOC, even at 1.2% concentration. The three novel gel retarded bands that appeared after dissociation of the initial complexes remained resistant to 1.2% DOC. Taken together, the results suggested that each of the DNA–nuclear extract complexes, derived from synchronized or preapoptotic G1 cells, contains multiple and potentially distinct cellular components.

Super-shift gel retardation assays were then performed to identify partner proteins in the E2F family of complexes (Philpott and Friend, 1994). pRb is a critical factor for E2F function and both its phosphorylation state and expression are inhibited by TGF-β1 in the preapoptotic cells (Fan et al., 1996). Therefore, anti-pRb monoclonal antibody (XZ161) was used to detect pRb in complexes derived from either the preapoptotic G1 or normal synchronized G1 cells. Interestingly, anti-pRb antibodies produced a significant shift only in complex VI of the four identified E2F-specific DNA–protein complexes from the preapoptotic nuclei (Fig. 5A, lanes 2,4). However, when the antibody was added to the synchronized G1 nuclear extracts, complexes VI, III and a significant percentage of complex II were shifted (Fig. 5A, lanes 1,3). The results suggested that complexes II and III contain pRb in normal G1 and this interaction was lost in G1 preapoptotic cells.

Other pRb family members such as p107 and p130 are also known to interact with members of the E2F family (Dyson, 1998). In fact, E2F-1, -2, and -3 can interact with pRb, but not with p107 (Lees et al., 1993). E2F-4 and -5 both associate preferentially with p107 and p130, although each can also interact with pRb (Moberg et al., 1996). Therefore, E2F-1, -2 and -3 as well as p107 and p130 antibodies were used in immuno-shift assays to investigate their presence in complexes from both G1 cell cycle phases. Our results showed that E2F-1, -2, and -3 are abundantly present in complexes III, IV and VI, and to a minor extent in complex II. This suggests that complexes I, II and V contain primarily E2F-4 and -5 (Fig. 5A, lanes 5-10). In contrast to pRb, the increased E2F activity did not alter the pattern of E2F association with p107 or p130. Both p107 and p130 were present in all the complexes that were derived from either the preapoptotic or synchronized G1 cells (Fig. 5B, lanes 7-10). However, complex V shifted less dramatically in the preapoptotic cells (Fig. 5B, lanes 2,8,10). Moreover, p130 antibody completely shifted complexes I, II and VI and a significant portion of complexes III and IV (Fig. 5B, lanes 2,8,10). Taken together, the data indicate that E2F-3 contributes significantly more than E2F-1 and E2F-2 to the increased E2F-specific DNA-binding activity observed in the preapoptotic G1 cells. Interestingly, p107 and p130 contributed substantially to complexes IV and VI that were derived from preapoptotic cells, which contained significant levels of E2F-1, -2 and -3.

It has been demonstrated that DP-1 and DP-2 associate with E2F-1, -2, and -3 in vivo and the complexes can activate transcription of E2F-specific sites (Bandara et al., 1994; Krek et al., 1993; Wu et al., 1995). Yet, such heterodimers preferentially form complexes with pRb, but not p107 and p130, which are the predominant pRb family members present in the E2F-DNA-binding complexes (Hijmans et al., 1995; Sardet et al., 1995). Therefore, to characterize formation of E2F–DP complexes and thus their potential for transcriptional activation in preapoptotic cells, anti-DP-1 and -DP-2 antibodies were used for super-shift gel retardation analysis. As shown in Fig. 5B, DP-1 exhibits maximal association with E2F in synchronized G1 cells but less so in preapoptotic cells (lanes 1-4). In both G1 cell populations, DP-2 exhibited a similar capacity for complex formation with some E2Fs, shifting an equivalent percentage of complexes I and IV or II and III, respectively (Fig. 5B, lanes 1-3). In contrast, complex VI, although completely shifted in synchronized G1 cells, showed only a slight decrease in preapoptotic cells with anti-DP-2. Although total E2F–DNA binding activity is significantly increased in preapoptotic cells, the extent of complex formation with DP-1 and -2 was reduced.

In addition to interacting with the pRb and DP family members, E2F-1 can bind to the oncogene MDM2, both in vitro and in vivo, and the formation of such complexes appears to increase E2F-dependent transcription (Martin et al., 1995). MDM2 can also physically interact with pRb and p53, inhibiting the pRb growth regulatory function and p53-dependent transcription, as well as increasing the degradation of p53 (Xiao et al., 1995). Thus, to determine whether the increase of unbound E2F in preapoptotic cells results in increased levels of interaction between E2F and MDM2, gel shifts were performed using anti-MDM2 antibodies. Our results indicated that the MDM2 antibody shifted all the complexes more completely in the synchronized G1 cells (Fig. 5B, lanes 1,11). In contrast, only complexes IV, V and VI derived from the preapoptotic cells were partially shifted by the MDM2 antibody (Fig. 5B, lanes 2,12). This suggests that MDM2 from preapoptotic cells and synchronized G1 cells interact differently with E2F. However, the increased E2F activity in preapoptotic G1 cells did not increase its level of association with MDM2.
Increased E2F activity mediates TGF-β1-induced apoptosis without S phase entry

A number of studies have reported that ectopic expression of E2F-1 induces rapid S phase entry and apoptosis (Shan and Lee, 1994; Asano et al., 1996). Therefore, we determined whether the increased E2F activity in TGF-β1-induced preapoptotic cells was sufficient for S phase entry. The cell cycle status of the remaining preapoptotic HuH-7 cells following 72 hours of TGF-β1 incubation was assessed by flow cytometry. Our results indicated that >90% of preapoptotic cells were in G1 phase (Table 1). Thus, even though similar dramatic increases in E2F-specific DNA-binding activity were observed in the same two complexes (V and VI) in both S phase and TGF-β1 treated preapoptotic cells, S phase entry did not occur (Fig. 2, lanes 1-3).

Removal of TGF-β1 leads to release of preapoptotic cells from G1 to S phase but does not completely inhibit apoptosis

To evaluate the potential irreversible apoptotic commitment induced by TGF-β1, the preapoptotic cells after 72 hours of exposure were released into normal culture medium containing 10% FBS. These preapoptotic cells rapidly entered S phase (47.9%) with some apoptosis (17%), resulting in a dramatic decrease of the G1 cell population from 91% to 21% by 15 hours post-release (P<0.001) (Table 1). G1 cells increased from 21% at 15 hours to 27% at 24 hours post-release; meanwhile, S phase cells increased slightly from 54% to 57% but with an additional 9% loss of the preapoptotic cells to apoptosis. By 48 and 72 hours post-release, the G1 cell population increased to 45%, and S phase cells decreased to 40%. Interestingly, the G2/M phase cell population was not significantly different at any time post-release, reflecting a constant ~7% of the total cells present by 24 hours post-release. In fact, after the initial post-release loss, ~7% of the remaining preapoptotic cells died every 24 hours after removal of TGF-β1. Taken together, the results suggest that E2F-mediated S phase entry was dependent on the removal of inhibitory signals but also the presence of appropriate cell cycle regulators for entry into S phase. A significant percentage of the cells arrested in G1 with TGF-β1 were irrevocably committed to cell death, and a return to normal cellular function could not be achieved even after removal of the apoptotic stimulus.

Increased E2F activity in preapoptotic cells mediates transcription that is reduced by overexpression of pRb

More than 1240 cellular genes have been identified that contain E2F sites that contribute to transcriptional regulation (Ishida et al., 2001; Müller et al., 2001). Most studies have demonstrated that E2F is a positive mediator of transcription (Flemington et al., 1993; Ginsberg et al., 1994). However, some results indicate that E2F may also involve suppression of transcription (Johnson et al., 1993; Müller et al., 2001). Two CAT transcription reporter constructs were used to determine whether the increased E2F-DNA binding activity observed in our preapoptotic cell nuclear extracts could accelerate E2F-mediated transcription. The E2F1CAT construct contained the entire human E2F-1 promoter on a 1.5 kb ApaI fragment driving the CAT gene (Johnson et al., 1993). The 4XE2FCAT construct expressed CAT under control of an E2F-dependent test promoter with E2F-specific DNA binding sites (Ohtani and Nevins, 1994). HuH-7 cells were transiently transfected with

Fig. 5. Determination of proteins bound to E2F derived from nuclear extracts of G1 and preapoptotic cells. The nuclear extracts were prepared from synchronized G1 and TGF-β1-induced preapoptotic cells. Aliquots were mixed with a 32P-labeled E2F-specific oligonucleotide probe and antibodies to either pRb, E2F-1, -2, or -3 (A), as well as known E2F-binding proteins, DP-1 and -2, the pRb family members p107 and p130 and the oncogene MDM2 (B). The super-shifted DNA–protein complexes were analyzed by PAGE as described in Materials and Methods. The position and numerical designation of the 32P-labeled DNA–protein complexes are indicated on the left and right. The associated cell cycle phases of the nuclear extracts are shown at the top as well as the specific antibody used for the super-shift analysis. The numerical designations for the lanes referred to in the text are indicated at the bottom. G1, nuclear extract from G1 synchronized normal cells; Tβ, nuclear extract from TGF-β1 preapoptotic G1 cells.
either of the two different reporter constructs and a β-galactosidase-expressing reporter plasmid to control for transfection and expression efficiency. After 24 hours, the transfected cells were washed, and then incubated for 36 hours with either 1 nM TGF-β1 or no addition. Prior to harvesting, the dead cells were removed by gentle washing and the remaining attached cells harvested to determine CAT activity. Treatment of the transfected cells with 1 nM TGF-β1 for 36 hours resulted in an increase of 84.8% in CAT reporter gene expression with the E2F1CAT and 138.5% with the 4XE2FCAT constructs (Fig. 6A). In contrast, the β-galactosidase control plasmid showed equivalent levels of expression irrespective of TGF-β1 treatment (data not shown). This data, coupled with the northern and western blot analyses of E2F-1 transcript and protein levels, respectively, suggests that post-transcriptional mechanisms predominate control the abundance of this E2F factor during TGF-β1 expression irrespective of TGF-β1 treatment (data not shown).

We previously demonstrated that overexpression of pRb prevents the apoptotic cell death associated with increased expression of E2F-1 in HuH-7 cells (Fan et al., 1996). Thus, co-transfection experiments were carried out using pCMV.RB to overexpress pRb together with either the E2F1CAT or 4XE2FCAT and the β-galactosidase control plasmid. Post-transfection, the cells were treated as outlined above and CAT activity assessed after 36 hours of incubation in the presence or absence of 1 nM TGF-β1. Overexpression of pRb inhibited CAT expression from the co-transfected E2F1CAT or 4XE2FCAT by 48.7% and 36.7%, respectively (Fig. 6B). This suggests that some of the E2F-controlled transcriptional activation induced by TGF-β1 can be partially reduced by simultaneous overexpression of pRb, confirming that the increased CAT expression results from TGF-β1-induced E2F activity in preapoptotic cells.

**Discussion**

It is apparent that a fine balance exists between levels of active pRb and E2F proteins that regulate cell cycle progression or exit (Harbour and Dean, 2000). In addition, it is well established that E2F is required for activation of certain cellular genes, and E2F-1 to -3 are essential for S phase entry (Wu et al., 2001). Our results suggest that within a normal cell cycle, increased levels of unbound or ‘free’ E2F in G1 are a prerequisite for the initiation of S phase, in which E2F is released from hypophosphorylated pRb (Ezhevsky et al., 2001). However, we determined that E2F-DNA-binding activity was significantly increased in TGF-β1-induced preapoptotic G1 cells compared with synchronized G1 in normal cells. In fact, the increased E2F activity did not appear to lead to S phase entry, but rather apoptosis. To address this dichotomy, we have assumed that E2F-mediated S phase entry depends on the availability of G1/S cellular mediators. If all the necessary players required for S phase entry are expressed at an appropriate level, increased E2F activity typically promotes S phase entry. In their absence, E2F expression directs cells into the apoptotic pathway (Harbour and Dean, 2000; Dyson, 1998). Thus, modulation of G1/S regulatory proteins can prevent S phase entry, and simultaneously induce apoptosis in G1 phase. In fact, we propose that TGF-β1 promotes apoptosis in G1, upsetting the fine balance between cell replication and cell death. In support of this, TGF-β1 can inhibit expression of a set of cell cycle regulator proteins that include cdk4 (Ewen et al., 1993), cyclin D1 (Ko et al., 1995), cyclin A (Slingerland et al., 1994), cyclin E and pRb (Fan et al., 1995; Fan et al., 1996). TGF-β1 also inactivates cyclin E/A-ckd2 by inducing p27kip1 (Mal et al., 1996), and inactivates cyclin E/ckd2 by inducing p21Cip1 (Reynisdottir et al., 1995) and cyclin-A and cyclin-E-associated histone H1 kinase activity (Reddy et al., 1994). In fact, the cyclin D/Cdk4 and cyclin E/Cdk2 kinase activities directly regulate the avidity of pRb for the E2Fs, either by increasing or decreasing binding, respectively (Ezhevsky et al., 2001). Finally, cyclin E and CDC25A are required for efficient E2F induction of S phase (Vigo et al., 1999).

There is no compelling evidence that a transient S phase entry actually exists prior to apoptosis. In fact, our previous studies of cell cycle kinetics in asynchronous cell populations suggest that it does not. TGF-β1 arrested more than 90% of
cells in the G1 phase and continuous exposure to the cytokine resulted in apoptosis without cell cycle progression (Fan et al., 1996). When these preapoptotic G1 synchronized cells were released from TGF-β1 and placed in normal growth medium for 15 hours, S phase cells increased by 47.9%. However, 17% of the released preapoptotic cells ultimately underwent apoptosis. The increase in these two cell populations accounted for greater than 90% of the loss observed in the G1 population. This implies that the loss of these preapoptotic cells represents a portion of the TGF-β1-treated cultures that were irreversibly committed to apoptosis and did not recover even under favorable growth conditions.

It is probable the apoptosis that occurred after removal of TGF-β1 was due primarily to cells already committed to cell death, as the characteristic apoptosis-associated nuclear morphologic changes were observed microscopically immediately after releasing the cells (data not shown). However, we cannot rule out the possibility that the apoptosis occurs rapidly after S phase entry. Several lines of evidence indicate that deregulated expression of E2F-1, -2 and -3 does induce S phase entry and apoptosis (Shan and Lee, 1994; Vigo et al., 1999). However, our data suggest that TGF-β1-induced apoptosis occurs predominately in G1 phase. If we consider that this is a primary process, then apoptosis induced by deregulated expression of E2F in S phase would be a secondary process. In fact, the dramatically increased E2F-DNA binding activity in the G1 preapoptotic cell population did not result in progression to S phase, but rather apoptosis. Additionally, there were similar increases in two E2F-specific DNA complexes relative to G1 phase in both normal S phase and preapoptotic cells; yet two complexes, which were identified in normal cells were absent and replaced by two unique complexes in the preapoptotic cells. The differences in E2F-specific DNA–protein complexes identified in nuclear extracts from S phase and preapoptotic cells further suggest that the increased E2F activity had not induced S phase, but rather a new apoptotic state.

The novel complexes identified in the preapoptotic state may, in part, explain the lack of effective progression to S phase even in the presence of greatly elevated E2F–DNA-binding activity. For example, the unique complexes I and IV are predominately E2Fs interacting with the two related pRb family members, p107 and p130, which are now thought to be the primary regulators of E2F-dependent cellular proliferation (Hurford et al., 1997; Takahashi et al., 2000). In fact, fibroblasts from pRb-deficient (pRb–/–) mouse embryos exhibited little alteration in expression patterns of E2F target genes as well as normal cell cycle regulation. Thus, p107 and p130 control the progression of the cell cycle through E2F association in the absence of functional pRb.

Alternatively, the increased nuclear E2F-DNA-binding activity observed may be a contributing factor to the observed G1 arrest. Both E2F-4 and -5 are actively exported from the nucleus during cell cycle entry, and this export appears to be required for exiting G1 (Gaubatz et al., 2001). Moreover, E2F-4 and E2F-5-deficient embryonic fibroblasts are unable to undergo pocket protein-mediated G1 arrest although they proliferate normally (Gaubatz et al., 2000). As the G1 arrest induced by pocket proteins is dependent on their ability to form E2F-containing transcription-repression complexes (Zhang et al., 1999), the data implies that E2F-4 and E2F-5 mediate pocket protein-dependent transcriptional repression during G1. This suggests that the unique complex I found in the preapoptotic cells comprised predominately of E2F-4 and E2F-5, and their pocket protein partners p107 and p130 prevent exit from G1 by maintaining transcriptional repression of factors required for S phase entry. In fact, keratinocytes treated with TGF-β1 also accumulate an E2F-4/p130 complex that with histone deacetylase HDAC1 directly represses cdc25A promoter activity, compromising progression through G1, and thus promoting cell cycle arrest in a quiescent state (Iavarone and Massagué, 1999).

E2F-mediated p53-dependent apoptosis has been extensively studied and suggests that wild-type p53 and E2F-1 cooperate to induce apoptosis in cultured cells (Wu and Levine, 1994). In transgenic pRb-knockout (pRb–/–) animals, the massive apoptosis associated with increased levels of unbound E2F-1 resulting in embryonic death is suppressed in certain tissues in fetuses from pRb+/+/p53–/+ mice (Clarke et al., 1992; Morgenbesser et al., 1994). However, dual knockout pRb+/+/pE2F-1–/– mice indicate that in the absence of E2F-1, although marked suppression of p53-dependent apoptosis is observed, embryonic death resulting from apoptosis still occurs (Tsai et al., 1998). This data suggests a significant role for other E2F family members in mediating this process.

E2F-3 also appears to have a direct role in mediating apoptosis in the absence of pRb, as shown in pRb/pE2F-3-deficient mice. In these animals, the concurrent loss of E2F-3 activity prevented both p53-dependent and -independent apoptosis observed in pRb-deficient animals (Ziebold et al., 2001). Interestingly, there was no reduction in cell number or evidence of apoptosis in the fetal livers of the pRb/pE2F-3-deficient mice in contrast to the pRb–/– mice. The dramatic increase in E2F-1 and E2F-3 binding activity coupled with the significantly decreased pRb levels in TGF-β1-treated cells suggests that the unique complexes identified in the preapoptotic cells may also promote apoptosis via transcriptional activation.

Increased E2F activity in TGF-β1-induced preapoptotic cells was found to increase E2F-related transcriptional activation. The increase of unbound E2F in preapoptotic cells, due to the loss of pRb binding, can directly activate expression of another set of apoptosis-related genes (Sofer-Levi and Resnitzky, 1996; Müller et al., 2001). For example, c-Myc can induce apoptosis in different types of cells (Evan et al., 1995), and its expression appears to be mediated, in part, by the transcription factor E2F-1. In fact, the c-myc P2 promoter activation by E2F-1 induces efficient transcription of c-myc, and this transcriptional activity can be abrogated by overexpression of pRb (Oswald et al., 1994). Recently, microarray analysis has demonstrated that, in the absence of de novo protein synthesis, the E2Fs directly induce the expression of several key regulators of apoptosis including the effector caspases 3 and 7, as well as the cytochrome-c-binding protein Apaf-1 (Müller et al., 2001). This suggests that the increased E2F activity observed in the preapoptotic cells could directly induce apoptosis via transcriptional activation of apoptosis-associated genes. However, this is most likely a secondary event in TGF-β1-induced apoptosis. Recently, we investigated the mechanism by which the bile acid ursodeoxycholic acid inhibits TGF-β1-induced apoptosis in primary rat hepatocytes and HuH-7 cells
transcriptional activation and apoptosis, underscoring its overexpression of pRb can significantly inhibit E2F-mediated cytochrome effector caspases that are activated subsequent to mitochondrial expression of the cytochrome-mediated transcriptional activation is directly responsible for HuH-7 cells. It remains to be established whether the E2F-DNA-binding activity and stimulates E2F-mediated transcriptional activation (Phillips et al., 1997; Phillips et al., 1999), although DNA binding appears to be important in Saos-2 cells, which do not express wild-type pRb or p53. Our studies showed that TGF-β1 incubation leads to significantly increased E2F-DNA-binding activity and stimulates E2F-mediated transcriptional activation and apoptosis but not proliferation in HuH-7 cells. It remains to be established whether the E2F-mediated transcriptional activation is directly responsible for expression of the cytochrome-c-binding protein Apaf-1 and effector caspases that are activated subsequent to mitochondrial cytochrome c efflux. Nevertheless, our studies indicate that overexpression of pRb can significantly inhibit E2F-mediated transcriptional activation and apoptosis, underscoring its critical role in regulating the life and death pathways associated with E2F.

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