Apaf-1 Is a Mediator of E2F-1-induced Apoptosis*

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E2F-1 is capable of promoting both cell cycle progression and apoptosis. The latter is important for suppressing untoward expansion of proliferating cells. In this study, we investigated its underlying mechanisms. E2F-1-induced apoptosis was accompanied by caspase-9 activation and inhibited by a specific inhibitor of caspase-9 in K562 sublines overexpressing E2F-1. E2F-1 enhanced the expression of Apaf-1 without the cytosolic accumulation of cytochrome c. Apaf-1-deficient melanoma cell lines were resistant to E2F-1, indicating that Apaf-1 is an essential element of E2F-1-mediated apoptosis. Finally, we isolated the promoter region of the Apaf-1 gene and found a putative binding site for E2F. A chromatin immunoprecipitation assay revealed that E2F-1 bound to Apaf-1 promoter upon E2F-1 overexpression, suggesting that Apaf-1 is under transcriptional regulation of E2F-1. These data demonstrate a novel mechanism of apoptosis in which an increase in Apaf-1 levels results in direct activation of caspase-9 without mitochondrial damage, leading to the initiation of a caspase cascade.

E2F is a family of transcription factors that control G1/S transition of eukaryotic cells by regulating the expression of various growth-related genes (1). Target genes of the E2F family include those encoding enzymes for DNA synthesis (DNA polymerase α, thymidine kinase, thymidylate synthase, dihydrofolate reductase, and ribonucleotide reductase), regulators of DNA replication (HsOrc1, Cdc6, MCM5, MCM6, and proliferating cell nuclear antigen), and components of the cell cycle machinery (Cdc2, Cdk2, cyclins A, D1, D2, and E, E2F-1, E2F-2, pRB, p107, and the Myc and Myb families) (2). Among E2F family members, E2F-1 is unique in its ability to induce apoptosis, which may play a role in the cellular homeostasis of multicellular organisms (3–5).

In response to mitogenic stimuli, E2F-1 is induced in quiescent cells and promotes both cell cycle progression and apoptosis (6). The apoptosis-inducing ability of E2F-1 is important for suppressing untoward expansion of proliferating cells and, thus, provides an internal defense mechanism against tumor development. The importance of E2F-1-induced apoptosis under physiological conditions is clearly demonstrated by spontaneous development of multiple tumors in mice lacking E2F-1 (7, 8). In addition, it has been reported that deregulation of E2F-1 activity contributes to enhanced proliferation and resistance to cytotoxic drugs in human melanoma cells (9). E2F-1 is also involved in the accelerated apoptosis of hematopoietic progenitor cells, which is considered the major mechanism of bone marrow failure in myelodysplastic syndrome (10). To clarify the molecular basis of these diseases, it is essential to understand the precise mechanisms of E2F-1-induced apoptosis.

There are a few reports dealing with the mechanisms of E2F-1-mediated apoptosis. First, Bates et al. (11) reported that E2F-1 induces transcriptional activation of ARF, which stabilizes p53 by sequestering MDM2, a ubiquitin ligase for p53. The stabilization of p53 results in facilitation of p53-dependent apoptosis. Obviously, this is not the sole mechanism of E2F-1-induced apoptosis, because E2F-1 can trigger cell death in p53-deficient tumors, including most leukemia cell lines (12). p53 is also shown to be dispensable for E2F-1-mediated cell death by genetic approaches (13). Second, the lack of NF-κB activation has been described as a mechanism of increased sensitivity of E2F-1-overexpressing cells to apoptotic stimuli through the death receptor pathways (14). However, this mechanism is not applicable to all cell types, because E2F-1 can induce apoptosis in a death receptor-independent manner in certain settings. Most recently, three groups demonstrated the involvement of the p53 homologue p73 in E2F-1-mediated apoptosis (15–17). Although p73 is known to cause apoptotic cell death, its underlying mechanism is still unclear (18). In view of the fact that p73 is also a transcription factor, it is possible that other direct mediators act downstream of p73 in E2F-induced apoptosis.

To explore the mechanisms of E2F-1-induced apoptosis, we established K562 sublines that can overexpress E2F-1 conditionally. Using this system, we found that E2F-1 was capable of activating caspase-9 to initiate the caspase cascade without mitochondrial damage. We further demonstrated that the activation of caspase-9 was caused by up-regulation of Apaf-1, which is a direct transcriptional target of E2F-1.

MATERIALS AND METHODS

Cell Lines and Cell Culture—The human immature myeloid cell line K562 was provided by Dr. Carmen B Lozzio (University of Tennessee Medical Center) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The human melanoma cell lines Malme-3M and SK-MEL-5 were purchased from ATCC (Manassas, VA). G-361 and Colo 679 were obtained from the RIKEN Cell Bank (Tsukuba, Japan), and MMAc was provided by Dr. Hiroshi Katayama (Katayama Dermatology Clinic, Gunma, Japan).

Reagents—All of the chemicals were purchased from Sigma unless otherwise noted.
Apaf-1 Is a Mediator of E2F-1-induced Apoptosis

The involvement of Apaf-1 in the mitochondria-dependent caspase pathway is supported by the finding that the loss of Apaf-1 activity leads to the blockage of E2F-1-induced apoptosis. This suggests that Apaf-1 may play a crucial role in the regulation of cell death induced by E2F-1.

Transient Transfection and Fluorescence Microscopy

To study the localization of Apaf-1 and mitochondria in transfected cells, we used transient transfection and fluorescence microscopy. The cells were transfected with a plasmid encoding a fusion protein consisting of Apaf-1 and a green fluorescent protein (GFP). The fluorescence was detected under a confocal microscope, and the localization of Apaf-1 and mitochondria was determined.

Measurement of Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential was measured using a MitoCapture mitochondrial transmembrane potential detection kit. The cells were stained with MitoCapture and analyzed using a fluorescence microscope. The data were analyzed using a software program to determine the mitochondrial transmembrane potential.

Apaf-1 Is a Mediator of E2F-1-induced Apoptosis

Apaf-1 is a key component of the mitochondrial apoptosis pathway, and its role in E2F-1-induced apoptosis was confirmed in this study. The results provide new insights into the mechanisms of E2F-1-induced apoptosis and the role of Apaf-1 in this process.
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Apoptosis Is a Relatively Late Onset Event in E2F-1-overexpressing Cells—To investigate the mechanisms of E2F-1-induced apoptosis, we established a K562 subline (KpEf1), which overexpresses full-length E2F-1 in the presence of IPTG (E2F-1) and a mock-transfected control (Mock) were cultured in the presence of 5 mM IPTG for 72 h and subjected to immunoblotting for E2F-1 expression and cell cycle analysis at the indicated time points. The positions of endogenous E2F-1 (End) and transgene (Trans) are shown with arrows. The molecular sizes are slightly different between endogenous and transgenic E2F-1 because of phosphorylation and acetylation. The data shown are representative of multiple independent experiments using different clones established at the same time.

following rabbit polyclonal antibodies (all purchased from Santa Cruz Biotechnology except anti-c-Kit): anti-E2F-1 (C-20), anti-E2F-2 (C-20), anti-E2F-3 (C-18), anti-E2F-4 (C-108), anti-E2F-5 (E-19), and anti-c-Kit (Oncogene Research, Boston, MA). After incubation at 4 °C for 2 h, the mixtures were further rocked with 20 μl goat anti-rabbit IgG-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) for 1 h, and the immune complexes were recovered in a magnetic separator. The immunoprecipitants were washed three times each with four different buffers, followed by elution with 50 μl NaHCO3 and 1% SDS. The eluents immune complexes were recovered in a magnetic separator. The immuno.

E2F-1 specifically induces cell cycle progression and apoptosis simultaneously but with different kinetics, and the induction of apoptosis is a relatively late onset event.

Involvement of Caspases-9, -6, and -3 in E2F-1-induced Apoptosis—To determine the pathways of apoptotic signal transduction activated by E2F-1, we examined the effects of specific inhibitors of caspases on E2F-1-induced apoptosis. KpEf1 cells were pretreated with cell-permeable caspase-binding peptides and then cultured in the presence of IPTG. The percentage of cells undergoing apoptosis was measured by calculating sub-G1 fractions on DNA histograms (Fig. 2, A and B) and DNA fragmentation ELISA (Fig. 2C) after 72 h. E2F-1-induced apoptosis was significantly suppressed by a general caspase inhibitor VAD as well as specific inhibitors for caspases-9, -6, and -3.

To confirm the activation of caspases during E2F-1-mediated apoptosis, we examined the expression and cleavage of caspases in E2F-1-overexpressing cells by immunoblotting. As shown in Fig. 3, the amount of procaspase-9 (48 kDa) decreased after 24 h of culture with IPTG in KpEf1 cells with active fragments of caspase-9 (37 kDa/17 kDa) appearing after 48 h. We simultaneously measured the activity of caspase-9 using colorimetric assay kits and found that it indeed increased in E2F-1-overexpressing cells in accord with the processing of procaspase-9 (data not shown). With similar kinetics, procaspases-6 (34 kDa) and -3 (32 kDa) were processed into a large active subunit (17 kDa) in these cells. No apparent effect on other initiator (caspase-8 and -2) and executioner (caspase-7) caspases was observed. These results suggest that E2F-1 activates caspase-9, which in turn processes caspases-6 and -3 to initiate a caspase cascade, leading to apoptotic cell death.

E2F-1-induced Caspase-9 Activation Coincides with Up-regulation of Apaf-1—The late onset of caspase-9 activation indicates that E2F-1 is not directly involved in the process but can initiate a caspase cascade through transcriptional regulation of the molecules that activate caspase-9. The candidates for such molecules include CARD-containing adapters, Apaf-1 (28) and Nod-1/CARD4 (29, 33), and an inhibitor of apoptosis-binding factor Smac/DIABLO (30, 34). We screened the expression of these genes in E2F-1-overexpressing clones by Northern blotting and found that E2F-1 increased the abundance of Apaf-1 mRNA after 24 h of culture (Fig. 4A). In addition, Nod-1/CARD4 mRNA was transiently up-regulated in an E2F-1-dependent manner, and the level of Smac/DIABLO transcript increased over time in both mock-transfected and E2F-1-overexpressing cells. To verify the induction of Apaf-1 by E2F-1, we examined the expression of Apaf-1 protein in E2F-1-overexpressing clones by Western blotting. Apaf-1 protein was barely detectable in untreated KpEf1 cells but was readily induced 48 h after the addition of IPTG (Fig. 4B).

It has been shown that Apaf-1 activates caspase-9 by interacting with procaspase-9 and promoting its oligomerization and cleavage in the presence of DATP and cytochrome c (35, 36). When mitochondria are damaged by certain apoptotic stimuli such as anticancer drug treatment, cytochrome c is released from the mitochondria into the cytosol, where it triggers oligomerization of Apaf-1 and activation of caspase-9 (23, 37). We therefore examined whether E2F-1 induces cytoplasmic translocation of cytochrome c in association with the increase in Apaf-1. Cytosolic proteins were serially isolated from E2F-1-overexpressing cells and subjected to immunoblotting for the evaluation of cytochrome c content. As shown in Fig. 4B, E2F-1 did not increase the amount of cytochrome c in the cytosol up to 96 h of culture, whereas adriamycin immediately induced a cytosolic accumulation of cytochrome c in these cells. To corroborate the result of immunoblotting, we examined the intracellular distribution of cytochrome c using confocal laser mi-
In untreated KpEf1 cells, cytochrome c was stained in a perinuclear punctate pattern indicative of intramitochondrial localization (Fig. 4 C, Control). Intramitochondrial localization of cytochrome c was confirmed by co-labeling with mitochondrial markers. During apoptosis triggered by adriamycin, the distribution of cytochrome c became completely diffuse, reflecting the translocation of cytochrome c into the cytosol (Fig. 4 C, ADR). In contrast, the pattern of cytochrome c distribution remained the same as that of the control in E2F-1-overexpressing KpEf1 cells up to 48 h of E2F-1 induction (Fig. 4 C, E2F-1).

Most if not all cells retained identical patterns, even after 72 h of culture when there was the initial signs of cell death, indicating that cytochrome c translocation is not a prerequisite for apoptosis following E2F-1 activation. Next, we measured mitochondrial transmembrane potential using a rhodamine-derivative dye to assess mitochondrial damage. As shown in Fig. 4 D, mitochondrial transmembrane potential was not severely affected during E2F-1-induced apoptosis, although it was lost in dead cells at day 3. These data suggest that the E2F-1-induced up-regulation of Apaf-1 is not a direct result of mitochondrial damage or massive cytosolic accumulation of cytochrome c. It is highly likely that Apaf-1 mediates E2F-1-induced apoptosis as a transcriptional target of E2F-1.

Finally, we investigated why E2F-1 did not induce cytoplasmic translocation of cytochrome c in KpEf1 cells. Upon DNA damage, p53 is stabilized by phosphorylation and induces transcriptional activation of Bax, which in turn acts on mitochondrial membrane to trigger the release of cytochrome c (38). We therefore examined the expression of p53 and Bax in KpEf1 cells. As shown in Fig. 5 A, p53 was not detectable in this cell line even after the induction of E2F-1 overexpression. We found that the lack of p53 protein was attributable to the defect of mRNA expression caused by a point mutation of the p53 gene (data not shown). Consistent with the absence of p53, the amount of Bax was not increased by E2F-1 in KpEf1 cells (Fig. 5 A). Furthermore, we also demonstrated the absence of mitochondrial translocation of Bax using confocal microscopy. Bax was detected in both the cytoplasm and the nucleus in untreated KpEf1 cells, whereas it accumulated in the cytoplasm and became co-localized with mitochondria after adriamycin treatment (Fig. 5 B). The change in Bax distribution was not observed after E2F-1 overexpression up to 72 h, indicating that Bax is not directly involved in cell death caused by E2F-1. These data may explain why cytochrome c release does not occur following E2F-1 overexpression in this cell line.
Apaf-1-defective Cells Are Relatively Resistant to E2F-1-induced Apoptosis

To confirm the involvement of Apaf-1 in E2F-1-induced apoptosis, we examined whether E2F-1 could elicit apoptosis in Apaf-1-defective cells. For this purpose, we first screened the expression of Apaf-1 in human melanoma cell lines, in some of which Apaf-1 is inactivated because of hypermethylation of an enhancer element of the Apaf-1 gene (39), by immunoblotting. Among the five cell lines examined, the Apaf-1 level was very low in G-361 and below the detection limit in SK-MEL-5 (Fig. 6A). E2F-1 was transiently overexpressed in these cell lines by transfecting a bicistronic vector that induces simultaneous expression of E2F-1 and GFP. At 72 h after transfection, we determined the percentage of GFP-positive cells undergoing apoptosis by fluorescence microscopy. The data from three independent experiments are summarized in Fig. 6B. The inducibility of apoptosis by E2F-1 was significantly lower in the two cell lines with little or no expression of Apaf-1 than in the other three melanoma cell lines with intact Apaf-1 expression. There was no difference in the proportion of apoptotic fraction among the five cell lines when an empty pIRES2-EGFP vector was transfected (Fig. 6B). These results indicate that E2F-1-induced apoptosis is at least in part mediated through Apaf-1-dependent pathways.

E2F-1 Binds to a Putative E2F-binding Site of Apaf-1 Promoter—Finally, we examined whether Apaf-1 is directly under the transcriptional regulation of E2F-1. To this end, we isolated the putative promoter region of the Apaf-1 gene by a data base-oriented approach (Fig. 7A). The promoter activity and E2F-1 responsiveness of the isolated region were confirmed by transient transfection of the pCAT basic vector containing PCR-amplified sequences between nucleotides −1136 and +34 into KpEf1 cells (data not shown). This region was found to be GC-rich; The percentage of G and C for the entire sequence is 66%, and the ratio of observed/expected CpG is 0.79. In partic-
Apafl-1 Is a Mediator of E2F-1-induced Apoptosis

In this study, we investigated the mechanisms of E2F-1-induced apoptosis using K562 sublines that can overexpress Apaf-1 conditionally. When E2F-1 was overexpressed in these cells, apoptosis was readily induced after 72 h, following a transient increase in the S phase at 12 h. This confirms the notion that E2F-1 promotes cell cycle progression and apoptosis simultaneously to suppress uncontrolled expansion of proliferating cells (3–6, 12).

We found that E2F-1 was capable of activating caspase-9 to initiate a caspase cascade in the absence of mitochondrial damage. The activation of caspase-9 is usually triggered by the release of cytochrome c from damaged mitochondria in response to certain apoptotic stimuli such as anticancer drugs, ultraviolet radiation, and serum deprivation (40). Subsequently, cytochrome c binds to Apaf-1 and, in association with adenine nucleotides, facilitates a conformational change of Apaf-1 into a CARD-containing adapter that recruits procaspase-9 by a homophilic interaction involving CARDs, which results in catalytic autoactivation of caspase-9. However, E2F-1 activates caspase-9 through a different pathway; it bypasses the translocation of cytochrome c from mitochondria to cytosol and directly induces autoactivation of caspase-9 via the increase in intracellular Apaf-1 levels. The involvement of other direct activators of caspase-9, including a CARD-containing adapter Nod-1/CARD4 (29, 33) and an inhibitor of apoptosis-binding factor Smac/DIABLO (30, 34), is to be determined because E2F-1 also modulates the expression of these molecules. Up-regulation of Apaf-1 is also observed during apoptosis caused by the adenoviral oncogene E1A, but it occurs as a secondary event of cytoplasmic accumulation of cytochrome c and therefore is distinct from that of E2F-1 (41). Although the mecha-
Fig. 7. E2F-1 binds to Apaf-1 promoter upon overexpression. A, the sequence of the 5′-untranslated region of the Apaf-1 gene is shown. Position +1 indicates the 5′ end of the full-length cDNA. The boundary of Exon 1 is indicated by a horizontal bar. Putative binding sites of known transcription factors are underlined, and a CpG island is boxed. The arrows correspond to the sequences used for PCR primers for chromatin immunoprecipitation assay. The nucleotide sequence shown here has been deposited in the DDBJ/EMBL/GenBank™ data base under accession number AB070829. B, chromatin suspensions were prepared from KpEf1 cells before (T-0) and after 24 h (T-24) of culture with IPTG and subjected to immunoprecipitation with the indicated antibodies. The resulting precipitants were subjected to PCR using a specific primer pair corresponding to the nucleotide positions of −618 to −597 and −428 to −407 of the Apaf-1 promoter. PCR was carried out in the presence of [32P]dCTP, and the amplified products were visualized by autoradiography after 8% polyacrylamide gel electrophoresis. The representative data of 30 PCR cycles are shown. No DNA, PCR was done without DNA templates. Input, prior to the first wash, 200 μl of the supernatant was saved for each time point and used for PCR after proteinase treatment and ethanol precipitation.
nism by which caspase-9 is activated by the increase in Apaf-1 is at present unclear, a high concentration of Apaf-1 may increase the probability of physical interaction between pro-caspase-9 molecules even in the absence of mitochondrial damage, resulting in oligomerization and subsequent autoactivation of procaspase-9. This hypothesis is substantiated by studies in which forced expression of Apaf-1 solely promoted apoptosis in human myeloid leukemia cell lines (42, 43).

Finally, we provide evidence that Apaf-1 is a direct transcriptional target of E2F-1 by analyzing the 5’-untranslated regions of the Apaf-1 gene. This finding is consistent with the results of a recent effort to identify E2F-responsive genes by global analysis of gene expression using high density oligonucleotide arrays; Apaf-1 is one of the newly identified E2F-1-inducible genes (44). Interestingly, E2F-2 and E2F-3 did not up-regulate Apaf-1 in this analysis, which is consistent with the fact that E2F-1 has the strongest ability in promoting apoptosis among E2F family members (5). However, there are some reports suggesting that other E2F proteins, especially E2F-2 and E2F-4, are also capable of inducing apoptosis (45, 46). The mechanisms underlying apoptosis induced by E2F-2 and E2F-4 may be different from that of E2F-1, because E2F-2 and E2F-4 cannot transactivate Apaf-1 (44, 47). Because E2F-4 was not able to induce apoptosis in our system (data not shown), it is not likely that E2F-2-mediated apoptosis involves p53-dependent pathways.

The structure of the Apaf-1 promoter is characteristic; it lacks either a typical TATA motif or initiator elements and is devoid of canonical binding sites for known transcription factors except E2F, Sp-1, and p53. Instead, the Apaf-1 promoter lacks either a typical TATA motif or initiator elements and is unable to induce apoptosis in our system (data not shown), it is not likely that E2F-2-mediated apoptosis involves p53-dependent pathways.

In conclusion, these data demonstrate a novel mechanism of apoptosis in which an increase in Apaf-1 levels induced by E2F-1 results in direct activation of caspase-9 without mitochondrial damage, leading to the initiation of a caspase cascade. Because both E2F-1 and Apaf-1 are frequently deregulated in various pathologic conditions, this finding may contribute to a better understanding of the pathophysiology of many diseases.

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Apaf-1 Is a Mediator of E2F-1-induced Apoptosis

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