E2F1 belongs to the E2F family of transcription factors. Seven different E2F members (E2F1 through E2F7) (1–4) and three DRF proteins (DP) (5) have been identified so far. As a heterodimeric complex with the DP-1 protein, E2F1 binds to promoters containing E2F-responsive elements and activates transcription through its C-terminal activation domain. Initial studies implicated E2F1 in the control of cell-cycle progression in response to DNA damage. E2F1 protein levels are also regulated by the ubiquitination-dependent proteasome degradation. The epitope recognized by the ubiquitin-proteasome pathway is located in the carboxyl terminus, near the acidic activation domain of E2F1, resulting in deacetylation of the E2F1-p300 acetyltransferase. This process is negatively affected by phosphorylation of the retinoblastoma gene product pRb, which is mainly mediated by the cyclin D1/cyclin-dependent kinase-4 at the G1/S transition (6). A second level of control of E2F1 activity concerns the regulation of its DNA-binding ability. Indeed, E2F1 interacts directly with cyclin A, which results in phosphorylation of E2F1 in the G1 phase, causing down-regulation and degradation of E2F1 binding to DNA (12, 13).

A final level of regulation of E2F1 relates to the control of its abundance by a number of mechanisms. After mitogenic stimuli, one or more E2F species activate(s) the E2F1 promoter in late G1, resulting in an increase in E2F1 RNA and protein synthesis. Conversely, transcription of the E2F1 gene decreases in late S, because of the negative regulation of the DNA-binding activity of E2F1–3 by cyclin A/cyclin-dependent kinase-2 (14, 15).

E2F1 protein levels are also regulated by the ubiquitination-dependent proteasome degradation. The epitope recognized by the ubiquitin-proteasome pathway is located in the carboxyl terminus, near the acidic activation domain of E2F1 (16, 17). Both post-translational modifications, such as phosphorylation by the transcription factor IIH (TFIH) kinase and protein-protein interactions with pRb have been shown to increase E2F1 stability through the inhibition of the ubiquitination process (18).

Distinct modalities of E2F1 regulation might be responsible for opposite outcomes of its activation, cell-cycle progression, or apoptosis. A prompt response to genotoxic stresses has been reported to induce stabilization of E2F1 through its phosphorylation by the ataxia-telangiectasia mutated (ATM) kinase, the ATM and RAD3-related (ATR) kinase (19) and the checkpoint kinase 2 (20, 21). We recently demonstrated that DNA damage also causes the acetylation-dependent activation of E2F1 apoptotic potential by boosting E2F1-driven transcription of the proapoptotic target gene p73 (22). Although E2F1 can be acetylated by both p300/CREB-binding protein (CBP)-associated factor (P/CAF) and CREB-binding protein/P300 acetylases in vitro at the same lysine residues (23, 24), the relationship between this post-translational modification and the stabilization of E2F1 in response to DNA damage has not been clarified.
been explored. We describe here a specific role for P/CAF in the acetylation-induced accumulation of E2F1 in response to DNA damage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—Human fibroblast cell lines AT-TERT and HFF-TERT, mouse embryo fibroblasts (MEFs) 3T3 ABL−/−, 3T3 P53−/−, and 3T3 E2F1−/−, U2OS and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with heat-inactivated 10% fetal bovine serum. Human glioblastoma cell line T98G was cultured in RPMI 1640 medium with heat-inactivated 10% fetal bovine serum, sodium pyruvate, and non-essential amino acids. MEFs 3T3 ABL−/− and 3T3 P53−/− were kindly provided by Dr. J. J. J. Wang. AT-TERT and HFF-TERT were kindly provided by Dr. J. Y. J. Wang and Dr. L. Yamasaki. MEFs 3T3 E2F1−/− were kindly provided by Dr. L. Yamashita. Transfections were performed either with LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions or with the standard CaPO4 method.

**Plasmids**—The wild-type E2F1 expression vector was generated by subcloning the PCR-generated full-length E2F1 cDNA into the HAPCDNA3 vector. The expression vectors pCDNA-HA-E2F1-S31/A (HA-E2F1-S31/A) and pCDNA-HA-E2F1-K117,12,125R (HA-E2F1-R) were generated by PCR-mediated site-directed mutagenesis and verified by sequencing. The PMCV E2F1, the wild-type and ΔHAT (Δ S 579–606) PCI P/CAF and the wild type and ΔHAT (LY-RH) PCI p300 expression vectors were kindly provided by V. Sartorelli and P. L. Puri (25).

**Immunoprecipitations and Immunoblotting**—The following antibodies were purchased from Santa Cruz Biotechnology: anti-E2F1 (C-20), anti-hemagglutinin (HA) monoclonal (F-7) and polyclonal (Y-11), anti-green fluorescent protein (GFP) (FL), anti-actin (I-19), and the agarose-conjugate anti-HA monoclonal (F7). Monoclonal anti-acetyl-lysines were purchased from Upstate Biotechnology. Cells were lysed with RIPA lysis buffer (50 mM Tris, pH 7.6, 1% Nonidet P-40, 140 mM NaCl, 0.1% SDS), and the insoluble pellet was discarded after centrifugation. Protein concentration was determined by the BCA protein assay reagent (Pierce). Extracts were immunoprecipitated with the indicated antibodies and protein A/G plus (Santa Cruz Biotechnology).

**Pulse-chase**—Subconfluent T98G cells in 10-cm dishes were transfected with 4 μg of either HA-E2F1-WT or with HA-E2F1-R. 12 h after transfection, cells were trypsinized and replated to normalize for the efficiency of transfection. 24 h after transfection, cells were starved for 1 h in methionine-cysteine-free medium and subsequently incubated with 200 μCi/ml of Premix (containing [35S]methionine-cysteine, Promix-Amersham) for 30 min. Medium with an excess of unlabelled methionine-cysteine (1 mg/ml) was used to stop the labeling. Medium was then added, and cells were collected at the indicated times. The 35S-labeled HA-E2F1 in the anti-HA immunoprecipitate from each time point was quantified by PhosphorImager and normalized to that of the zero time point.

**Cycloheximide Chase**—Transfected U2OS cells were seeded in 35-mm dishes. Two days after transfection, cells were treated with 2 μM doxorubicin for 8 h, and then Me3SO-solubilized cycloheximide (Sigma) was added to the culture medium at a final concentration of 50 μg/ml for the indicated times.

**RESULTS**

**E2F1 Is Induced in Response to DNA Damage in the AT-Tert Human ATM-defective Cell Line**—Genotoxic stress has been shown to induce both phosphorylation and acetylation of E2F1 (19, 22). To investigate the relative contribution of these two post-translational events in E2F1 stabilization induced by genotoxic stresses, we first assessed the role of the ATM/ATR pathway in E2F1 activation in response to DNA damage. To this purpose, we analyzed ATM-defective primary fibroblasts derived from a patient affected with ataxia-telangiectasia (AT) and immortalized by the stable expression of the catalytic subunit of telomerase (AT-TERT) (26). We found that treatment of AT-TERT cells with doxorubicin still leads to stabilization of the E2F1 protein, although to a lesser extent and with a delayed kinetics, with respect to control HFF-TERT cells (Fig. 1a). To rule out the hypothesis that the ATM and RAD3-related kinase ATR might be responsible for the delayed accumulation of E2F1 observed in our cells, we compared the kinetics of stabilization of wild-type E2F1 versus the E2F1 mutant (HA-E2F1-S31/A) that cannot be phosphorylated by both ATM and ATR. We observed that exogenously expressed wild-type E2F1 was already accumulated after 1 h of doxorubicin treatment, whereas induction of the exogenously expressed HA-E2F1-S31/A mutant still occurred, although it was delayed (Fig. 1b). Taken together, our data imply that additional post...
translational events, other than ATM/ATR-dependent phosphorylation, might contribute to E2F1 stabilization in cells exposed to the DNA-damaging drug doxorubicin.

E2F1 Stabilization in Response to DNA-damaging Drugs Is Independent of P53 and cAbl—Searching for additional pathways involved in DNA-damage-dependent stabilization of
E2F1, we analyzed the role of p53. Indeed, increasing evidence suggests that the integrity of the Rb pathway could influence the activity of p53 and vice versa. E2F1 cooperates with p53 to induce apoptosis by increasing ARF transcription and thereby blocking murine double minute 2 (MDM2)-mediated p53 degradation (27). In addition, it has been shown that p53 interacts directly with the E2F1/DP1 complex through the cyclin A-binding domain located in the N terminus of E2F1. Cyclin A inhibits E2F1 binding to p53 in vitro and abrogates the ability of E2F1 to cooperate with p53 to induce apoptosis in vivo in response to DNA damage (28). Moreover, p53 lies upstream of E2F1 in the apoptotic pathway induced by UV treatment in mouse keratinocytes (29). As shown in Fig. 2a, endogenous E2F1 accumulates to a very similar extent in p53−/− MEFs as compared with wild-type MEFs exposed to either cisplatin or doxorubicin, thus excluding a role for this kinase in this context.

**E2F1 Acetylation Is Required for Its Stabilization in Response to DNA Damage**

As shown in Fig. 2b, endogenous E2F1 protein accumulates in response to both treatments in cAbl−/− MEFs, thus excluding a role for this kinase in this context.

**P/CAF Hat Activity Is Required for E2F1 Stabilization in Response to DNA Damage**

E2F1 can be acetylated in vitro at lysine residues 117, 120, and 125 by P/CAF and, to a lesser extent, p300 acetyltransferases (23, 24). Acetylation of these residues leads to increased DNA-binding affinity, induction of its transcriptional activity, and accumulation of the protein. Because doxorubicin treatment leads to the accumulation of acetylated E2F1 protein species (Fig. 3a; Ref. 22), we hypothesized that DNA damage-induced acetylation might be responsible for the E2F1 stabilization we observed in the absence of ATM/ATR-dependent phosphorylation. To assess whether E2F1 acetylation is required for its stabilization after genotoxic stress, we first analyzed the behavior of a non-acetylatable E2F1 mutant protein (HA-E2F1-R) (24). As shown in Fig. 3b, exogenously expressed wild-type E2F1 accumulates 5 h after doxorubicin treatment, whereas the HA-E2F1-R mutant is not accumulated at all at the time points investigated. Doxorubicin treatment does not change cellular levels of E2F1 mRNA (data not shown; Refs. 24, 34). Therefore, we examined
whether acetylation might affect protein half-life in cells exposed to DNA-damaging drugs. The levels of exogenously expressed wild-type E2F1 and HA-E2F1-R proteins were monitored in a pulse-chase experiment, either in the presence or the absence of doxorubicin. Fig. 3c shows that HA-E2F1-WT half-life is significantly prolonged in response to doxorubicin treatment, whereas the half-life of the HA-E2F1-R mutant remains unaffected. Altogether, these results indicate that acetylation of E2F1 is required to stabilize the protein in response to DNA damage.

**Different Role for P/CAF and p300 in E2F1 Stabilization after DNA Damage**—In vitro studies indicate that P/CAF is more efficient in acetylating E2F1 with respect to p300. However, the relative role of the two acetyltransferases in influencing E2F1 stability following genotoxic stress was not determined. To address this issue, we performed co-immunoprecipitation experiments to investigate the ability of P/CAF and p300 to form complexes with E2F1 in response to DNA damage. As shown in Fig. 4a, immunoprecipitation of exogenously expressed FLAG-tagged P/CAF in E2F1–/− MEFs treated with doxorubicin results in increased levels of co-immunoprecipitated exogenous HA-E2F1 (lanes 1–2). Likewise, exogenously expressed HA-E2F1 co-immunoprecipitates FLAG-P/CAF only in treated cells (Fig. 4a, lanes 3–4). Unlike P/CAF, p300 complex formation with E2F1 is not modulated by doxorubicin treatment (Fig. 4a, lanes 5–6).

To investigate the relative contributions of P/CAF and p300, and, specifically, of their HAT activity in E2F1 stabilization in response to DNA damage, we performed a cycloheximide chase on transfected U2OS cells treated with doxorubicin. As shown in Fig. 4b, exogenously expressed P/CAF (lanes 4–6, row a) and p300 (lanes 10–12, row a) do not alter E2F1 half-life in untreated cells. Conversely, in doxorubicin-treated cells, the half-life of E2F1 is significantly prolonged in the presence of P/CAF (compare lanes 4–6 to lanes 1–3, row c). Stabilization of E2F1 is likely due to the activation of the P/CAF acetyltransferase activity, because the P/CAF-HAT mutant (lanes 7–9 versus lanes 1–3, row c), deleted of the acetyltransferase domain, is unable to stabilize E2F1, when compared with wild-type P/CAF. On the other hand, p300 (lanes 10–12 versus lanes 1–3, row c) only slightly increased E2F1 half-life in the presence of doxorubicin, whereas its HAT-defective mutant (lanes 13–15 versus lanes 1–3, row c) showed behavior similar to the control empty vector both in treated and untreated cells.

**DISCUSSION**

Many transcription factors have been identified as substrates for P/CAF and p300/CREB-binding protein (35). Particularly, acetylation regulates the transcriptional activity of p73 and p53 in response to genotoxic stress (36). We recently demonstrated that DNA damage induces E2F1 apoptotic potential (22). This is achieved by the selective relocalization of E2F1 from cell-cycle progression genes to the promoter of the apoptotic p73 gene, where E2F1 accumulates into transcriptionally active complexes that include the acetyltransferase coactivator P/CAF. We found that E2F1 acetylation is required in this context (22).

Here we report that in addition to specifying its transcriptional potential, DNA damage-induced acetylation of E2F1 is also important for its accumulation. Indeed, the finding that E2F1 is stabilized in the ATM-defective cell line AT-TERT strongly suggests that additional pathways, other than ATM/ATR-dependent phosphorylation, are required to stabilize E2F1 in response to genotoxic stress. We ruled out a role for p53 and cAbi, two of the major transducers of DNA-damage signaling, in inducing E2F1 accumulation in this context. We found that DNA damage-induced acetylation of E2F1 is the major determinant of its accumulation. We further assessed the relative role of the two acetyltransferases P/CAF and p300, both previously reported to be involved in E2F1 acetylation. Although E2F1 binding to p300 is unaffected by DNA damage, the affinity between P/CAF and E2F1 is strongly increased in doxorubicin-treated cells. Accordingly, only the HAT activity of P/CAF, and not that of p300, is required to induce a prolongation of E2F1 half-life in the presence of genotoxic stress.

The mechanisms that lead to the increased stability of the acetylated E2F1 protein still remain elusive. Acetylation of p53 has also been reported to induce its stabilization by preventing its MDM2-dependent ubiquitination (37). Similarly to p53, E2F1 is degraded through the ubiquitin-proteasome pathway. Phosphorylation at the Ser-403 in S phase by the TFIIH kinase targets E2F1 for its rapid ubiquitination and degradation (18). One mechanism might be that acetylation of E2F1 competes with the TFIIH-dependent phosphorylation, thus subtracting the fraction of E2F1 targeted for elimination. Furthermore, binding to Rb also protects E2F1 from its ubiquitination (14, 15). A differential interaction of E2F1 with Rb in response to DNA damage may impart distinct fates to the protein. This possibility is also suggested by the recent identification of an additional binding site for E2F1 on Rb, which putatively controls E2F1-apoptotic functions and is regulated by DNA damage (38). These observations and the results we reported here, together with the fact that Rb functions are regulated by acetylation as well (39), suggest that acetylation might coordinately control Rb/E2F1 interaction in response to DNA damage and, in this way, E2F1 stability and apoptotic activity.

In conclusion, the findings reported here support previous observations (22), indicating that E2F1 acetylation plays a pivotal role in the recruitment of its apoptotic potential and are consistent with a two-step model of E2F1-driven apoptotic response to DNA-damaging agents. In this model, DNA damage first enhances P/CAF-dependent acetylation of E2F1, resulting in its stabilization and formation of stable protein complexes with P/CAF. The increased levels of acetylated E2F1 are then available for its selective recruitment onto the p73 gene promoter to trigger the apoptotic response. Whether this mechanism is impaired in human cancers, frequently associated to genetic impairments of the Rb/E2F1 pathway, or might be exploited to improve the therapeutic index of genotoxic anticancer drugs, will represent the goal of our future investigations.

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