c-Myc-induced Aberrant DNA Synthesis and Activation of DNA Damage Response in p300 Knockdown Cells*

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We previously showed that in quiescent cells, p300/CBP (CREB-binding protein) family coactivators repress c-myc and prevent premature induction of DNA synthesis. p300/CBP-depleted cells exit G1 early and continue to accumulate in S phase but do not progress into G2/M, and eventually they die of apoptosis. Here, we show that the S-phase arrest in these cells is because of an intra-S-phase block. The inappropriate DNA synthesis that occurs as a result of forced expression of c-myc leads to the activation of the DNA damage response as evidenced by the phosphorylation of several checkpoint related proteins and the formation of foci containing γ-H2AX. The activation of checkpoint response is related to the induction of c-myc, as the phosphorylation of checkpoint proteins can be reversed when cells are treated with a C-Myc inhibitor or when Myc synthesis is blocked by short hairpin RNA. Using the DNA fiber assay, we show that in p300-depleted cells initiation of replication occurs from multiple replication origins. Chromatin loading of the Cdc45 protein also indicates increased origin activity in p300 knockdown cells. Immunofluorescence experiments indicate that C-Myc colocalizes with replication foci, consistent with the recently reported direct role of c-Myc in the initiation of DNA synthesis. Thus, the inappropriate S-phase entry of p300-depleted cells is likely to be because of C-Myc-induced deregulated origin activity, which results in replicative stress, activation of a DNA damage response, and S-phase arrest. Our results point to an important role for p300 in maintaining genomic integrity by negatively regulating c-myc.

In eukaryotic cells, initiation of DNA replication takes place from multiple replication origins on each chromosome, providing flexibility for the large eukaryotic DNA to replicate efficiently. However, control mechanisms exist to ensure that DNA replication occurs only once per cell, and when such mechanisms fail, checkpoint responses are activated to monitor the genome integrity and inhibit replication until DNA damage has been repaired (for review, see Ref. 1). In response to DNA damage, eukaryotic cells activate ATM1/Chk2 and ATR/Chk1 kinase pathways to arrest the cell cycle and allow time to repair DNA. Both ATM and ATR control cell cycle checkpoint signals by phosphorylating a number of checkpoint-related proteins including Chk1 and Chk2 and p53 (for review, see Refs. 2 and 3).

p300 and CBP are two highly related and conserved nuclear phosphoproteins that function as transcriptional coactivators, and by interacting with a large number of sequence-specific transcription factors they integrate diverse signal transduction pathways in the nucleus. Because of their intrinsic histone acetyltransferase activity, they also acetylate nucleosomal histones and, thus, link chromatin remodeling with transcription (for review, see Ref. 4). Recent studies suggest that p300/CBP plays an important role in DNA replication and the activation of DNA damage response by interacting with several proteins involved in DNA replication and/or by acetylating proteins involved in DNA metabolism (see “Discussion”). We recently discovered that ablation of p300 or CBP in quiescent cells leads to induction of c-myc that in turn induces DNA synthesis in the absence of growth factor stimulation (5, 6). DNA tumor virus-encoded transforming proteins such as adenovirus E1A and SV40 large T antigen also bind to and inactivate p300/CBP and thereby induce c-myc and S phase in quiescent cells without mitogen stimulation (5, 7). Although premature DNA synthesis in p300 down-regulated cells allows them to exit G1, they accumulate in S phase but fail to progress into G2/M and ultimately die of apoptosis (6). Failure of p300/CBP-depleted cells to traverse beyond S phase is not related to the lack of growth factor stimulation because the addition of serum to p300/CBP-depleted cells also leads to increased c-Myc synthesis and an S-phase block (6).

c-myc is known to induce DNA synthesis by transcriptionally targeting genes involved in DNA replication (for review, see Ref. 8). However, recent studies suggest that c-myc can also directly control the initiation of DNA replication without transcriptionally targeting the replication-related genes (9). c-Myc can interact with DNA replication origin complex and function as a replication licensing factor (9). Excess c-Myc can promote elevated DNA synthesis, and the deregulated origin activity can induce DNA damage response by the ATM pathway (9, 10). Because p300/CBP prevents inappropriate DNA synthesis by negatively regulating c-myc and p300/CBP knockdown results in the induction of c-myc both in quiescent and proliferating p300/CBP-depleted cells also leads to increased c-Myc synthesis and an S-phase block (6).

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3 The abbreviations used are: ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia and Rad3-related; Ad, adenovirus; CBP, cAMP-CREB-binding protein; PCNA, proliferating cell nuclear antigen; Chk1, Checkpoint homolog (Schizosaccharomyces pombe) 1; Chk2, check-point homolog 2; PARP-1, poly(ADP-ribose) polymerase family member 1; NBS1, Nijmegen Breakage syndrome gene 1; shRNA, short hairpin RNA; AdM4, adenovirus M4 vector; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; CldUrd, chlorodeoxyuridine; γ-H2AX, phospho-histone H2AX.
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cells (5, 6), it was of interest to determine whether the S-phase block that occurs in p300/CBP-depleted cells is related to the forced induction of c-myc and whether the DNA damage response is initiated in these cells. We now show that the inappropriate DNA synthesis that occurs in p300 knockdown cells as a result of c-myc induction is most likely because of uncontrolled replication origin activity. This c-myc-induced deregulation of DNA replication origin activity causes replication stress, an intra-S-phase block, and activation of a DNA damage response. These findings suggest an important role for p300 in maintaining genomic integrity by negatively regulating c-myc.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Growth conditions for the human MCF10A cells (11) were described earlier (5, 12). Conditions for serum starvation, serum stimulation, and cell cycle analysis were as described (5, 12). The adenovirus M4 vector (AdM4) carries a luciferase reporter cassette in E1A region in which four copies of the Myc binding sites were cloned upstream of the luciferase reporter gene (12). AdM4mut contains mutated Myc binding sites, and as a result the myc reporter activity of this vector is greatly reduced (12). Details of the two Ad vectors expressing p300 shRNAs, targeting two different regions of p300 (Adp300sh1 and -2), and the Ad vector expressing shRNA, targeting the luciferase gene, were described in a recent report (13).

Protein Expression, Promoter Reporter Assays, and Cell Cycle Analysis—Cells were lysed in Nonidet P-40 lysis buffer with protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor I and II (Sigma). About 50–120 μg of protein were subjected to Western immunoblotting as described earlier (14). Promoter reporter assays were carried out using MCF10A cells. Briefly, cells (4–6 × 10⁶ per well) grown as indicated in the legend to Fig. 5 were infected with AdM4 virus (2 plaque-forming units (pfu)/cell) along with Ad vectors expressing shRNAs (25 pfu/cell). The cells were harvested at the indicated time points in the figures, and the luciferase activity was measured from equal amounts of protein extracts as described (12). For cell cycle analysis the cells were harvested, trypsinized, washed in cold PBS, and fixed in 70% ethanol overnight. The cells were then incubated for 20 min in PBS containing 40 μg/ml propidium iodide and 0.5 mg/ml RNase A. Flow cytometry was carried out using a BD Biosciences FACSscan.

Cdc45 Chromatin Loading Assay—Chromatin, nuclear soluble, and cytosolic fractions were prepared as described by Menendez and Stillman (15). Briefly, cell pellets were resuspended in buffer A (10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, and protease inhibitors), and Triton X-100 (0.1%) was added and incubated on ice for 5 min. Nuclei were collected as a pellet (P1) by low speed centrifugation for 5 min at 3000 × g at 4 °C. The supernatant S1 was centrifuged at 20,000 × g for 20 min to remove insoluble aggregates and cell debris. Nuclei were washed once in buffer A and then lysed in buffer B (1 mM dithiothreitol, 3 mM EDTA, 0.2 mM EGTA, and protease inhibitors). Insoluble chromatin was collected by centrifugation at 1700 × g for 4 min to give rise to soluble nuclear fraction (S3) and pellet (P3). The pellet was washed once with buffer B, and the final chromatin pellet (P3) was resuspended in Laemmli buffer and sonicated for 15 s. Proteins released from chromatin (P3) and proteins present in nuclear soluble (S3) and cytoplasmic fractions (S1) were analyzed for the presence of Cdc45 using the Western immunoblotting procedure.

Immunofluorescence Studies—MCF10A cells were grown on coverslips, infected with Ad vectors, and processed for immunofluorescence studies. Growth conditions and duration of infection with Ad vectors are described in the legend to each figure. To block the c-Myc activity, cells were treated with 60 μM Myc inhibitor 10058-F4 ((Z,E)-5-(4-ethylbenzylidene)-2-thioxothiazolidin-4-one; Calbiochem) in the presence of DMSO for the duration of time indicated in the legends to Figs. 5 and 7 then processed for immunofluorescence studies. For immunofluorescence analysis, the cells were fixed with methanol/acetone (1:1) for 10 min, washed with PBS, permeabilized with 0.25% Triton-X100 in PBS, and blocked with 3% bovine serum albumin for 30 min then incubated with the primary antibodies at 1:500 dilution overnight. The coverslips were rinsed with PBS and then incubated with secondary antibodies (Alexafluor; Molecular Probes) for 1 h at room temperature. The coverslips were washed with PBS and mounted on the microscopic slides using mounting media with DAPI (Vector laboratories). For labeling with 5-bromodeoxyuridine (BrdUrd), cells were pulse-labeled with 50 μM BrdUrd (Sigma) for 1 h. The details of the antibodies used in all of the experiments reported in this paper are provided in the legends to the relevant figures. The images were captured using either a Nikon fluorescent microscope at 40× magnification or a Nikon EZ-C1 confocal microscope at 100× magnification, and the images were processed by Adobe Photoshop. For quantification of the immunostained cells, at least 100 cells from three different fields were counted, and the average values are shown.

DNA Fiber Assay—DNA spreads were prepared as described by Merrick et al. (16). Briefly, cells were first labeled with IdUrd (25 μM) for 30 min, washed, and then labeled with CldUrd (25 μM) for 30 min. Cells were then trypsinized and resuspended in ice-cold PBS at 2.5 × 10⁶ cells/ml. The labeled cells were diluted 1:10 in unlabeled cells, and 2.5 μl of cells were mixed with 7.5 μl of spreading buffer (0.5% SDS in 200 mM Tris-HCl, pH 7.4, 50 mM EDTA) on a glass slide. After 8 min the slides were tilted at −15 degree, and the resulting DNA spreads were air-dried, fixed in 3:1 methanol/acetic acid, and refrigerated overnight. The slides were then treated with 2.5 M HCl for 1 h, washed extensively with PBS, and blocked with 1% bovine serum albumin in TBST followed by incubation overnight with rat α-BrdUrd (detects CldUrd) and mouse α-BrdUrd (detects IdUrd) at 1:500 dilution. The slides were then rinsed three times with TBST and incubated for 1 h at room temperature with α-rat Alexafluor-594 and α-mouse Alexafluor-488 (Molecular Probes) at 1:500 dilutions. They were then rinsed with TBST and mounted with mounting media (Vectorshield; Vector laboratories). Microscopy was carried out using Nikon fluorescent microscope. The images for immunofluorescence and DNA fiber analysis were captured at 40× magnification and were processed using Adobe Photoshop.
DNA Synthesis Assay—To determine the radioactivity incorporated into DNA on a per cell basis, proliferating cells were infected with Ad vectors expressing shRNAs and harvested at various time points as indicated in Fig. 1D. Cells were then subjected to propidium iodide-based flow cytometry to determine the percentage of cells in S phase. In parallel, in the same experiment control and p300 shRNA-vector-infected cells (2 × 10^5 cells/well in 12-well plates) were pulse-labeled with 1 μCi of [3H]thymidine per well for 30 min before harvesting, and cells were collected at the same time points used for cell cycle analysis. The harvested cells were lysed with a DNA lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.5% SDS). The lysates were then passed through a 21-gauge needle several times and spotted on glass fiber discs, washed sequentially with 5% trichloroacetic acid and 95% ethanol (3 times each), and air-dried. The incorporated radioactivity was measured using a liquid scintillation counter, and the [3H]thymidine incorporation into DNA on a per cell basis was calculated by dividing the total CPM by the number of cells in S-phase fraction.

RESULTS

Down-regulation of p300 in Human Cells Leads to Accumulation of Cells in S Phase—The studies reported here were carried out in quiescent as well as proliferating cells to rule out the effect of serum on p300 depletion, c-myc induction, and the initiation of the DNA damage response. In studies reported here and those reported previously, we have used MCF10A cells, immortalized non-transformed human breast epithelial cells that can be readily growth arrested by serum starvation (11). These cells contain wild-type p53 and provide us with an ideal cell line to study the molecular aspects of serum-induced cell cycle regulation. Using antisense p300 and CBP constructs, we showed earlier that c-myc is induced in quiescent or proliferating MCF10A cells when p300 or CBP levels drop considerably (5, 6). Such cells accumulate in S phase but do not progress further.

To determine the effects of down-regulation of p300 in cycling MCF10A cells, we infected two sets of proliferating cells with two different Ad vectors expressing shRNAs that target two different regions of p300 (p300sh1 and p300sh2) for 18 h then monitored cell cycle progression using flow cytometry. As shown in Fig. 1A, the distribution of cells infected with an Ad vector expressing control shRNAs (specific for luciferase) showed a normal cell cycle profile, whereas the two sets of p300 shRNA-expressing cells showed an aberrant cell cycle pattern. For example, most of the p300sh1 and p300sh2 RNA-expressing cells exited G0/G1 and accumulated in S phase, and the number of cells in the G2/M fraction was reduced by about 2-fold as compared with control samples. These results were supported by a BrdUrd labeling experiment in which proliferating cells were infected with Ad vectors expressing p300 shRNAs then pulse-labeled with BrdUrd to visualize DNA synthesis. The BrdUrd-positive cells were identified using indirect immunofluorescence. Data shown in Fig. 1B indicate that about 70% of the p300-depleted cells were positive for BrdUrd, whereas only about 20% of the control cells were BrdUrd-positive (quantification made by counting BrdUrd-positive cells, see “Experimental Procedures”; quantification data are not shown). Fig. 1C shows that c-Myc protein levels were elevated in p300 shRNA-expressing cells as compared with control cells. Together, the above results suggest that p300 shRNA-expressing cells exited G0/G1 faster than control cells and accumulated rapidly in S phase but failed to progress further. Thus, when p300 is down-regulated, cells do not cycle normally and are blocked in S phase.

p300 Down-regulated Cells Accumulating in S Phase Develop an Intra-S-phase Block—A possible reason for the inability of the p300 shRNA-expressing cells to progress beyond S phase is that there may be incomplete DNA replication as a result of an intra-S-phase block. Exposure of cells to DNA-damaging conditions such as ionizing radiation or ultraviolet light arrests the bulk of DNA synthesis until the DNA damage is repaired (for review, see Ref. 17). To determine whether DNA synthesis is stalled in p300-depleted cells, cells were pulse-labeled with [3H]thymidine, and radioactivity incorporated into DNA was determined on a per cell basis as described under “Experimental Procedures.” In the experiment shown in Fig. 1D, a set of cells was used for measuring [3H] incorporation per S-phase cell at various times after depletion of p300, and a parallel set of cells was used to determine cell cycle distribution. The results shown in Fig. 1D show that the number of p300-depleted cells in S phase increased by about 2-fold by 18 h after infection of cells with p300 shRNA-expressing vectors. Although [3H]thymidine incorporation decreased with time in all samples, the incorporation of [3H]thymidine on a per cell basis decreased in the p300-depleted cells. For example, the 18-h control sample showed a 25% decrease of radioactivity as compared with 0h time point. In contrast, both p300 knockdown samples showed a 50% decrease in radioactivity. These results are at variance with cell cycle analysis, which showed a 2-fold increase of p300-depleted cells in S phase. At present we do not have a good explanation for this observation. One possibility is that although more replication origins in p300-depleted cells initiated DNA synthesis and entered S phase, these replicons stalled and accumulated as hypoactive origins. These stalled replication forks could account for S-phase accumulation and activation of the DNA damage response.

Chromatin Loading of Cdc45 Is Increased in p300 Knockdown Cells—To determine whether the p300 knockdown cells accumulating in S phase had evidence of increased origin activity, we measured the levels of the chromatin loading of Cdc45 protein. Cdc45 has been implicated in initiation of DNA replication, and chromatin-bound Cdc45 has been used as an indicator of replication origin activity (18–20). To determine the chromatin loading of Cdc45 in p300 down-regulated cells, chromatin-bound proteins were extracted from cells infected with vectors expressing control and p300 shRNAs for 12 and 16 h as described under “Experimental Procedures” and subjected to Western blotting. At this time point, there was a 2-fold increase in S-phase cells in the p300-depleted cultures (data not shown). Data shown in Fig. 1E indicate that there was a 2-fold increase in chromatin-bound Cdc45 and a 3–4-fold increase in total Cdc45 levels in nuclear fractions. These results suggest that the origin activity is increased in p300 knockdown cells.
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The DNA Damage Response Is Activated in p300-depleted Cells Accumulating in S Phase—Central to DNA damage signaling is a pair of related protein kinases, ATR and ATM, which are activated by DNA damage and recruited to the sites of DNA damage. After recruitment, they phosphorylate a number of substrates including Chk1, Chk2, and NBS. To determine whether the DNA damage response is activated in p300-depleted cells that accumulate in S phase, we assayed the phosphorylation of the proteins characteristic of the cellular response to DNA damage (1). Asynchronous MCF10A cells were infected with the two p300 shRNA vectors or the control virus for 18 h, then cell extracts were prepared and subjected to Western immunoblotting using antibodies specific for p300, CBP, c-Myc, and for the phosphorylated checkpoint proteins ATM, Chk1, and Chk2. As shown in Fig. 2A, p300 levels were reduced severalfold in both shRNA-expressing cells without affecting CBP levels. In agreement with our previous results, Myc protein levels were elevated, and p53 levels were reduced severalfold (6), consistent with a role for p300 in regulation of p53 levels (21). Importantly, both ATM and Chk2 were found to be phosphorylated at significant levels. An earlier study showed that p300 depletion in HeLa cells does not result in Chk1 phosphorylation, suggesting that ATR/Chk1 pathway is not active during p300 down-regulation (22). We have confirmed these results (data not shown). The above results indicate that the DNA damage response was activated in p300-depleted cells that accumulate in S phase. These cells contained elevated levels of c-Myc protein as compared with control cells (data shown in Fig. 1C). Thus, checkpoint activation in p300 knockdown-proliferating cells correlates with the increased Myc protein levels.

An early step in response to double-strand DNA breaks in mammalian cells is the phosphorylation of a histone variant H2AX at sites of DNA damage (for review, see Ref. 2). Genotoxic stresses such as stalled replication forks or DNA double-strand breaks can induce formation of microscopic foci containing thousands of molecules of phosphorylated H2AX (γ-H2AX (2)). To determine whether the p300 shRNA-expressing cells that accumulate in S-phase-form γ-H2AX-containing foci, p300 cells grown on coverslips were reacted with α-γ-H2AX antibody (18 h after vector infection) and examined for the formation of foci by fluorescent microscopy. About 60% of the p300 shRNA-expressing cells were found to be positive for γ-H2AX-containing foci, whereas a negligible number of
control cells showed such foci. These results suggest the presence of stalled replication forks and/or DNA double-strand breaks in p300-depleted cells (Fig. 2, B and C). Thus, we conclude that p300-depleted cells that accumulate in S phase activate checkpoint kinases.

Induction of c-myc in p300 Knockdown Quiescent Cells Correlates with the Induction of DNA Damage Response—To determine whether the induction of c-myc that occurs in p300-down-regulated cells correlates with induction of the DNA damage response, we monitored the kinetics of p300 down-regulation, activation of c-myc, and phosphorylation of DNA damage response proteins in p300-depleted quiescent cells. Serum-starved MCF10A cells were infected with Ad vectors expressing p300 shRNAs, and cells were harvested at the indicated time points shown in Fig. 3A. Cell lysates prepared from these cells were immunoblotted using relevant antibodies as shown in Fig. 3A. These data indicate that levels of p300 began to decrease beginning at the 12-h time point, whereas CBP levels remained constant. A large increase in Myc protein levels was observed by about 18 h. Phosphorylation of ATM, Chk2, and NBS1 (data not shown) was also observed beginning at around 18 h after infection of cells with Adp300 shRNAs. p53 levels dropped when p300 levels decreased, consistent with the role of p300 in maintaining normal p53 levels (21). The drop in p53 levels suggests that activation of the checkpoint in these cells is not driven by p53. Flow cytometric analysis of these cells indicated that more than 70% of p300 shRNA-expressing cells accumulated in S phase with fewer than 5% of the cells in G2/M (data not shown). Formation of γ-H2AX-containing foci was also determined in MCF10A cells at 6, 18, and 24 h post-infection with Adp300 shRNAs. As shown in Fig. 3B, no foci were visible at 6 h post-infection, whereas at 18 h post-infection, γ-H2AX-containing foci were readily observed (Fig. 3, B and C). In summary, the activation of c-myc in quiescent cells in response to p300 down-regulation correlated with activation of the DNA damage response.

Increased Number of Replication Origins Is Fired in p300-depleted Cells—In addition to inducing DNA replication by transcriptionally activating DNA replication-related genes, c-myc can also regulate DNA replication origin activity by directly binding to the origins (9). Therefore, we speculated that the activation of the DNA damage response in p300 shRNA-expressing cells could be because of firing of an increased num-

FIGURE 1. p300 knockdown proliferating MCF10A cells arrest in S phase (A and B), contain elevated c-Myc levels (C), and show an intra-S-phase block (D and E). A, flow cytometric analysis of proliferating MCF10A cells infected with Ad vectors Adp300sh1 or Adp300shRNA2 targeting two different regions of p300-coding sequences or Adluc-sh-targeting luciferase gene (see “Experimental Procedures”). Cells were infected at 25 plaque-forming units/cell with the Ad vectors as shown, and the cells were harvested after 18 h, then their DNA content was measured by flow cytometry as described under “Experimental Procedures.” B, BrdUrd incorporation into DNA of p300-depleted proliferating MCF10A cells. Cells grown on coverslips were infected with Ad vectors as above and were pulse-labeled with 50 μM BrdUrd for 1 h before processing for immunostaining. The cells were then fixed, and BrdUrd was immunoreacted with mouse α-BrdUrd antibody (BD Biosciences). Bound antibodies were visualized using a mouse antibody conjugated with Alexafluor-592 (red). DNA was detected by staining with DAPI (blue). C, the percentage of γ-H2AX positive cells were scored from three different fields, and the average was plotted with ± S.D. At least 100 cells were counted in each field.

FIGURE 2. Activation of the DNA damage response in proliferating p300 knockdown cells. A, phosphorylation of DNA damage response proteins Chk2 and ATM in p300-depleted cells. Proliferating MCF10A cells were infected with Ad vectors expressing Luc-sh, p300sh1, or p300sh2 as shown, and the cells were harvested after 18 h. Equal amounts of protein extracts were used for Western immunoblots to detect various proteins as indicated. Sources of antibodies used: α-p300 and α-actin (Santa Cruz), α-CBP (Upstate Biotechnology), α-ATM (Bethyl), α-pATM (Rockland), α-Chk2, α-pChk2, and α-p53 (Cell Signaling). B, formation of γ-H2AX containing foci in p300-depleted cells. Proliferating cells grown on coverslips were infected as above for 18 h, then the cells were immunoreacted with α-γ-H2AX antibody (Upstate). Bound antibodies were detected using a mouse antibody conjugated with Alexafluor-592 (red). DNA was detected by staining with DAPI (blue). C, the percentage of γ-H2AX positive cells scored from three different fields, and the average was plotted with ± S.D. At least 100 cells were counted in each field.
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**FIGURE 3.** A time course study shows depletion of p300, induction of c-Myc, and phosphorylation of Chk2 and ATM in quiescent cells (A) and the formation of γ-H2AX-containing foci (B). A, activation of checkpoint in quiescent cells. MCF10A cells were made quiescent by incubating them in culture media containing 0.2% serum for 48 h, then the cells were infected with Adp300sh1, Adp300sh2 (data not shown; see below), or Adlucsh and maintained in the starvation media as above. Cell extracts were made at the indicated time points.

DNA fiber assays that allow the visualization of firing replication origins, progression of replication forks, and terminations (for review, see Ref. 23). Serum-starved MCF10A cells were infected with the two Ad vectors expressing p300 shRNAs for 18 h and then labeled with IdUrd followed by CldUrd, each for 30 min each. After labeling, cells were collected and lysed on glass slides, and DNA fibers were spread as described (16). DNA fibers were immunostained to visualize CldUrd (red color tracks in Fig. 4A) and IdUrd-containing replicons (green color tracks in Fig. 4A, also see “Experimental Procedures”). The images obtained using fluorescent microscopy of the DNA fibers are shown in Fig. 4B. These data reveal dramatically different patterns for mock and p300 shRNA-expressing cells. For example, a strikingly higher number of replication origins appear to have been fired in both p300 shRNA 1- and 2-expressing cells as compared with control cells.

Furthermore, in p300 shRNA-expressing cells most DNA fibers showed green color tracks (arrows) that are interrupted frequently with red color tracks (arrowheads). A simple interpretation of these grossly altered labeling patterns of DNA in p300 knockdown samples is that initiation of DNA replication may have occurred from a significantly increased number of origins after the first pulse (shown as arrows in Fig. 4B) and that the replication forks from these origins terminated frequently (shown as arrowheads in Fig. 4B). We note here that because the images shown here were captured at low resolution (40× magnification), our analysis and interpretation of these tracks is limited. However, it is clear that the pattern of tracks that we observed for p300-depleted cells is strikingly different from that of the control samples.

Inhibition of c-Myc Activity or c-Myc Synthesis in p300 Knock-down Cells Reverses the Checkpoint Activation—Next, we determined whether the activation of the DNA damage response in p300-depleted cells is linked to elevated Myc levels and firing of an increased number of replication origins. To do...
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In this study, we blocked Myc activity using two strategies. In the first strategy, we blocked Myc activity using the Myc inhibitor 10058-F4 (see “Experimental Procedures” for further details). This inhibitor has been shown to block Myc binding to DNA by interfering in Myc-Max heterodimer formation. As a result, Myc shRNA did not interfere with Myc transcriptional activity without affecting Myc protein levels (24). To confirm whether the inhibitor blocks Myc transcriptional activation activity, we infected the proliferating cells with Adlucsh (control) or Adp300 shRNA vectors in the presence of 60 μM 10058-F4 for 24 h. After treatment with inhibitor, cells were harvested, and Myc protein levels were determined by Western blots. c-myc transcriptional activity was determined using an Ad vector-containing Myc-responsive promoter-luciferase reporter cassette (AdM4) described earlier (12). A control Ad vector that lacks the Myc binding sites was used as a negative control (AdM4mut). Fig. 5B shows that Myc protein levels remained steady when p300 shRNA-expressing proliferating cells were treated with the Myc inhibitor. As shown in Fig. 5A, the Myc inhibitor reduced the Myc activity only marginally in cells expressing control shRNAs. In p300 shRNA-expressing cells, Myc activity increased by about 4-fold, which was reduced to basal levels by the Myc inhibitor, indicating that the inhibitor was very effective in blocking the transcriptional activity of Myc. An increase in Myc activity was because of increased Myc binding to the Myc binding sites of the reporter construct because cells infected with AdM4mut showed only the basal activity (Fig. 5, A and C). Fig. 5B shows that treatment of p300 shRNA-expressing cells with the Myc inhibitor blocked the phosphorylation of Chk2 without affecting Chk2 protein levels.

The Myc inhibitor experiment was also performed with quiescent MCF10A cells in which the treatment with the inhibitor led to a complete block in Myc transcriptional activation (Fig. 5C) without altering Myc protein levels (Fig. 5D). Myc inhibition also blocked Chk2 phosphorylation that occurs in p300 shRNA-expressing cells.

In another strategy, shRNAs targeting p300 and c-myc were expressed simultaneously in proliferating cells, and then the activation of checkpoint was monitored by assaying the phosphorylation of the checkpoint related proteins. As shown in Fig. 5E, synthesis of the endogenous Myc in the absence of p300 shRNAs was almost non-detectable. Synthesis of Myc protein in p300 shRNA-expressing cells was also reduced by roughly 3-fold when shRNAs targeting myc and p300 were expressed simultaneously (Fig. 5E, quantification was based on visual inspection of a lighter exposure of the autoradiogram). Note that p300 shRNA-expressing cells contain significantly higher levels of Myc protein as compared with control cells because of induction of c-myc. As a result, Myc shRNA did not reduce Myc proteins to levels comparable to that of myc down-regulated control cells (Fig. 5E, lane 2). Nonetheless, the roughly 3-fold reduced Myc synthesis was effective in blocking the Chk2 phosphorylation (Fig. 5E). Thus, we conclude that the activation DNA damage response in p300 shRNA-expressing cells is related to c-myc overexpression. In summary, the above data suggest that Chk2 phosphorylation in p300-depleted cells is related to increase in Myc protein levels.

Formation of γ-H2AX-containing Foci in p300 shRNA-expressing Cells Is Blocked by the Myc Inhibitor—The Myc inhibitor also prevented the formation of γ-H2AX-containing foci in p300-down-regulated cells. To show this, we serum-starved MCF10A cells for 48 h, expressed the two p300shRNAs using Ad vectors, and incubated the cells in the presence of the inhibitor. At the end of the incubation, cells were fixed and stained with DAPI and immunoreacted with α-γ-H2AX antibody to visualize the foci. Data shown in Fig. 5F indicate that the γ-H2AX containing foci were visible only in p300 shRNA-expressing cells but not in p300 shRNA-expressing cells treated with the inhibitor. These results indicate that the stalled replication forks or double-strand breaks occurring in p300 shRNA-expressing cells is linked to Myc overexpression.

Myc Colocalizes with an Increased Number of Replication Foci Formed in p300 shRNA-expressing Cells—As stated before, studies have shown that myc plays a role in DNA replication by interacting with replication origins without transcriptionally targeting the replication related genes (9). To determine whether the increased number of replication origins activated in p300 shRNA-expressing cells contain Myc, replication foci were examined for the presence of Myc. Quiescent MCF10A cells grown on coverslips were infected with p300 shRNAs. The

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4 N. Sankar and B. Thimmapaya, unpublished results.
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FIGURE 5. Blocking c-Myc activity using a Myc inhibitor or reducing c-Myc protein levels using a Myc shRNA reverses the DNA damage response. A, inhibition of transcriptional activity induced in p300-depleted proliferating cells by the Myc inhibitor. Proliferating MCF10A cells were infected with Ad vectors expressing various shRNAs as described in the legend to Fig. 2A and infected 2 h later with an Ad vector containing an Myc-responsive promoter-luciferase reporter construct and a mutant version of AdM4 in which the Myc binding sites are mutated (AdM4 and AdM4mut, respectively, Ref. 12). Cells were then treated with an Myc inhibitor as described under “Experimental Procedures” for 18 h and harvested, and the luciferase activity in the cell lysates was quantified exactly as described in legend to Fig. 2A. C, inhibition of transcriptional activity in quiescent MCF10A cells infected with Ad vectors expressing shRNA. Cells were infected with two Ad vectors targeting p300 followed by the Myc inhibitor and luciferase activity in the cell lysates was quantified exactly as described in A. The experiment was carried out in triplicate, and the values from two different experiments are shown with ± S.D. D, inhibition of checkpoint activation by the Myc inhibitor in p300-depleted quiescent cells. Serum-starved MCF10A cells were infected with various Ad vectors for 18 h and treated with the Myc inhibitor as described in A, then harvested 18 h later. Myc, pChk2, and Chk2 levels were determined by Western immunoblotting. Details of the antibodies are as described in Fig. 2. A, E, down-regulation of Myc protein levels by Myc shRNA prevents the phosphorylation of Chk2. Proliferating MCF10A cells were infected with an Ad vector expressing Myc shRNA, and after 2 h the cells were again infected with Ad vectors expressing p300 or luciferase specific shRNAs. The cells were harvested 18 h later, then the protein extracts were subjected to Western immunoblotting to detect Myc and phosphorylated and unphosphorylated Chk2 levels. F, inhibition of the formation of the γ-H2AX-containing foci in p300-depleted quiescent cells by the Myc inhibitor. Serum-starved cells grown on coverslips were infected with Adp300shRNA1 or -2 for 18 h then treated with the Myc inhibitor as described above. Cells were then immunoreacted with α-γH2AX antibody. Detection of γ-H2AX was as detailed in Fig. 2C. The percentage of γ-H2AX positive cells is shown with ± S.D.
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We used two different shRNAs targeting two different regions of p300 (13) to ablate the expression of p300 and to study the effects of p300 ablation on G_{1}→S transition. The cell cycle effects we studied include development of an intra-S-phase block in S-phase-accumulating cells, induction of checkpoint activation, formation of γ-H2AX-containing foci, and localization of Myc in DNA replication foci. Data obtained using two p300 shRNA vectors in both quiescent and proliferating cells are in complete agreement, indicating that consequences of p300 knockdown is likely related to the overexpression of c-myc.

Our studies show that the elevated c-Myc present in p300 shRNA-expressing cells is primarily responsible for the observed effects on the induction of DNA damage response in cells that accumulate as a result of reduced levels of p300. Evidence includes the reversal of many of these effects when Myc levels were reduced by a Myc shRNA, or Myc activity was blocked by a Myc inhibitor. We previously showed that the S-phase entry of the p300 or CBP knockdown cells in the absence of growth factors could be reversed when the induction of c-Myc was prevented (5, 6). Consistent with this observation, we showed that activation of the DNA damage response (phosphorylation of checkpoint proteins) in S-phase-accumulating p300 knockdown cells can be prevented when Myc levels are reduced or the Myc activity was blocked by a Myc inhibitor (Fig. 5, B, D, and E).

Myc is a powerful mitogen and induces a large number of genes by directly binding to their promoters. Many of the Myc target genes are involved in the initiation and elongation of DNA replication and G_{1} and S-phase progression (for review, see Ref. 8). Forced expression of Myc in quiescent cells has been shown to induce S phase (for review, see Ref. 27) that was believed to be independent of Rb-E2F pathway in which E2F is released from Rb repressor complex as a result of Rb phosphorylation (28). However, a recent study shows that Myc can also regulate the DNA replication origin activity by directly binding to pre-replication complex proteins (9). Thus, Myc can induce DNA synthesis by inducing synthesis of proteins involved in the initiation of DNA synthesis as well as by directly interacting with replication origins. Using DNA fiber assays, we showed that in p300 shRNA-expressing cells DNA replication is most likely initiated from a large number of replication origins as compared with normal serum-stimulated cells. Using BrdUrd labeling followed by indirect immunofluorescence we showed that in p300 shRNA-expressing cells Myc is colocalized in a large number of the DNA replication foci. The colocalization of Myc in replication origins could not be observed when cells are treated with the Myc inhibitor. Thus, the elevated levels of c-Myc in p300 shRNA-expressing cells could contribute to the initiation of DNA synthesis both by inducing the synthesis of proteins relevant to the initiation of DNA replication as well as by interacting with replication origins directly. The presence of increased origin activity in p300 down-regulated cells is also suggested by our data, showing a 2-fold increase in Cdc45 in chromatin-bound fractions in p300 down-regulated cells. Our analysis of DNA fiber assays, although limited, suggests that in p300-depleted cells a large number of replication origins may have been activated.

cells (Fig. 6D). These cells were pulse-labeled with BrdUrd and immunoreacted with α-BrdUrd and α-c-Myc antibodies, and colocalization of c-Myc and BrdUrd was examined by confocal microscopy. c-Myc showed a diffused pattern in control cells, whereas most of the Myc in p300 knockdown cells was concentrated in the nucleus (Fig. 6E). Merging data showed that there was also no significant overlap between BrdUrd and Myc staining patterns in control cells. In contrast, there was a dramatic overlap between Myc and BrdUrd immunofluorescence patterns in p300 knockdown cells (Fig. 6E). Interestingly, both BrdUrd and the BrdUrd and c-Myc merged images showed a clustered appearance in p300 knockdown cells, indicating that the replication foci are arranged in a distinct pattern. The significance of these findings is not clear at present.

To determine whether co-localization of Myc in the replication foci is related to the elevated levels of Myc in p300-depleted cells, an Myc inhibitor experiment was performed. Proliferating cells expressing p300 shRNAs for 18 h were treated with Myc inhibitor, then pulse-labeled with BrdUrd, stained with DAPI, and immunoreacted with α-Myc or α-BrdUrd antibodies to visualize the relevant proteins. As shown in Fig. 7A, Myc could not be detected in replication foci in cells treated with the inhibitor. PCNA, a processivity factor for DNA polymerase-δ, binds to a number of proteins involved in DNA replication (for review, see Ref. 25). The presence of PCNA in the replication foci was also shown in p300-depleted cells using α-PCNA antibody. In Myc inhibitor-treated p300-depleted cells, the localization of PCNA was not seen, which further confirms the role of elevated c-Myc levels in activating an increased number of replication origins.

To confirm the co-localization of Myc and PCNA in the replication centers in p300 knockdown cells, immunofluorescence was examined under confocal microscopy. The confocal images shown in Fig. 7B suggest that more than 80% of the Myc-containing foci corresponded to BrdUrd-containing foci. The co-localization of PCNA containing foci with BrdUrd-containing foci was not as extensive as that of Myc and BrdUrd-containing foci, which might be related to stalled or inactive replication forks. Similar observations were made in other systems (26). This observation is also consistent with other results presented in this paper which suggest that although a large number of replication origins are activated in p300-depleted cells, they might be accumulating as hypofunctional origins.

DISCUSSION

In this paper we studied the effects of forced c-myc activation in human cells on DNA replication, when p300 levels are reduced. Our previously published data showed that when either p300 or CBP is down-regulated in quiescent cells, c-myc is induced, which in turn induces S phase (6). The p300- or CBP-depleted cells continue to accumulate in S phase but fail to progress into G_{2}/M and eventually die of apoptosis. We were interested to know the molecular basis of accumulation of these cells in S phase in p300/CBP-depleted cells and whether DNA damage response is activated in these cells and, if so, whether the activation of the DNA damage response is related to the induction of myc.

We used two different shRNAs targeting two different regions of p300 (13) to ablate the expression of p300 and to study the effects of p300 ablation on G_{1}→S transition. The cell cycle effects we studied include development of an intra-S-phase block in S-phase-accumulating cells, induction of checkpoint activation, formation of γ-H2AX-containing foci, and localization of Myc in DNA replication foci. Data obtained using two p300 shRNA vectors in both quiescent and proliferating cells are in complete agreement, indicating that consequences of p300 knockdown is likely related to the overexpression of c-myc.

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A

6h  
18h  
24h  
p300sh1
Quiescent cells

B

c-Myc  BrdU  DAPI  Merge

Lucsh  
p300sh1  
p300sh2  
Proliferating cells

D

G0/G1: 43  
S: 48  
G2/M: 9  
p300sh1

Lucsh  
P300sh

G0/G1: 47  
S: 43  
G2/M: 10

Lucsh  
P300sh

E

BrdU  c-Myc  DAPI  Merge

Lucsh  
p300sh1  

C

Bar graph showing BrdU-Myc positive cells for Luc-sh, p300sh1, and p300sh2.
For some reasons that we cannot explain at present, we did not observe a commensurate increase in \(^{3}H\)thymidine incorporation into DNA of p300 knockdown cells accumulating in S phase (Fig. 1D). One possibility is that the replication origins that are activated and entered S phase lose activity and accumulate as hypofunctional replication origins. Such origins may be either inactive or less efficient in supporting DNA synthesis after initiation. Consistent with this observation, we have never detected endoreduplication of DNA in p300 knockdown cells (6).\(^4\) Because we did not examine the DNA replication fork progression in these samples, we are unable to comment on the nature of the replication forks accumulating in S phase.

At present we do not know whether the increased origin activity in p300 down-regulated cells is because of activation of new origins or reinitiation from the normal origins that are activated but silenced as soon as DNA replication begins. Recent reports show that mammalian cells grown in culture undergoing replication stress activate new origins within a replicon cluster. These origins do not normally fire (dormant origins) but are activated despite the activation of the S-phase check points (29–31). So it is possible that the replication stress that occurs in p300 down-regulated cells leads to the activation of the dormant origins. Clearly further studies are required to determine the molecular basis of the deregulated initiation.

Currently we do not know the factors that contribute to the intra-S-phase block and checkpoint activation in p300 shRNA-expressing cells. Others have shown that overexpression of oncogenes such as cyclin E, cdc6, mos (32), ras (33), and myc (10, 34) induces DNA hyper-replication by activating multiple DNA replication origins (35). In p300-depleted cells DNA replication initiated from multiple origins may be terminated prematurely because of lack of sufficient levels of proteins essential for various steps in the coordinated progression of replication. As stated in the Introduction, p300/CBP by itself has been shown to regulate a number of proteins involved in DNA replication and repair by its physical association with them and/or by acetylation and modulation of their activity. For example,
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PCNA acts as a processivity factor for DNA polymerase-δ and DNA polymerase-ε. It has been proposed that PCNA recruits p300 to damaged sites to acetylate nearby histones, remodel chromatin to a more open state, and facilitate recruitment of other repair proteins to the damaged site (36). p300 acetylates Flap endonuclease-1 (Fen1) heavily in vitro, and acetylation is enhanced in vivo after UV damage (37). During DNA replication, Fen1 processes Okazaki fragments, and during DNA repair, it is involved in base excision repair (37). Acetylation of Fen1 by p300 results in a decrease in its DNA binding activity. Thymine glycosylase is an enzyme that repairs G/U and G/T mismatches. It has been shown that p300/CBP interacts with this repair protein and acetylates histones at or near the damaged site (38). Another study showed that p300 forms a complex with DNA polymerase-β (Polβ) in vitro; Polβ is involved in DNA base excision repair (39). Acetylation of polymerase-β by p300 led to down-regulation of its activity in vitro. The role of p300/CBP in the cellular response to DNA damage is likely to be more complex than that described above, as a recent large scale proteomic study showed that more than 700 proteins are phosphorylated after ionizing radiation (40). A recent study carried out in HeLa cells showed that Chk1 phosphorylation was dependent on ablation of both p300 and CBP (22). In contrast, in MCF10A, which are immortalized human breast epithelial cells, depletion of p300 alone was sufficient to activate the DNA damage response.

A number of studies have shown that overexpression of c-myc induces apoptosis (for review, see Ref. 41). We have shown earlier that p300/CBP depletion using antisense vectors induces apoptosis both in the presence and absence of serum (6). We confirmed that apoptosis occurs in p300-depleted cells, accumulating in S phase in which checkpoint activation is initiated. To detect the apoptotic response, we assessed the cleavage of PARP-1 by caspases, which is a prominent biochemical hallmark of apoptosis (42). When cell lysates were assayed 48 h after infection of cells with Ad vectors expressing p300 shRNAs, PARP-1 was cleaved into 89- and 27-kDa fragments, indicating that these cells are undergoing apoptosis (data not shown).

Results reported in this paper are also relevant to cell transformation by the DNA tumor virus tumor antigens such as the Ad E1A-transforming protein. Earlier reports have shown that E1A mutants such as RG3, and dl2–36 fail to transform rodent cells in culture (for review, see Ref. 43). We have shown that E1A induces c-myc in a p300 binding-dependent manner (14), and depletion of p300 also leads to c-myc induction, deregulated DNA synthesis, and induction of DNA damage response (this paper). Others have shown that E1A activates DNA damage response and sensitizes cells to DNA-damaging agents by accumulating p53 and apoptosis (44). Viral proteins such as E1B 19K or activated Ras protect cells from apoptosis and induce cell transformation (43). However, a role for forced c-myc expression in inducing DNA damage response in E1A-expressing cells has not been reported. Thus, p300 binding-dependent E1A-mediated induction of c-myc that is linked to deregulation of DNA replication may be an obligatory step in cell transformation.

As discussed above, the role of p300/CBP in the DNA damage response appears to be complex because of its histone acetyl transferase activity and its capacity to coactivate a large number of transcription factors, many of which are involved in DNA replication. Both p300 and CBP are mutated in several forms of cancers, but it is not clear whether the DNA damage response is activated and whether myc is deregulated in these cancer cells and whether these two aspects are linked. We have obtained preliminary data showing that a cancer cell line derived from HTCC cells in which the p300 is knocked out (45) shows evidence of activation of a DNA damage response. It will be interesting to determine whether various cancer cell lines in which p300 is mutated consistently show a DNA damage response.

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