NF-Y Mediates the Transcriptional Inhibition of the cyclin B1, cyclin B2, and cdc25C Promoters upon Induced G2 Arrest*  

Received for publication, July 10, 2000, and in revised form, October 31, 2000  
Published, JBC Papers in Press, November 28, 2000, DOI 10.1074/jbc.M006052200

Isabella Manni§, Giuseppina Mazzaro¶, Aymone Gurtner§§, Roberto Mantovani, Ulrike Haugwitz**, Karen Krause**, Kurt Engeland**, Ada Sacchi‡, Silvia Soddu‡, and Giulia Piaggio‡ ‡‡  

From the 2Laboratorio Oncogenesi Molecolare, Istituto Regina Elena, Rome 00158, the 1Dipartimento di Biologia Animale Università di Modena e Reggio, Modena 41100, Italy, and the **Medizinische Klinik II, Max-Burger-Forschungszentrum, Universitat Leipzig, Leipzig D-04103, Germany

During normal cell cycles, the function of mitotic cyclin-cdk1 complexes, as well as of cdc25C phosphatase, is required for G2 phase progression. Accordingly, the G2 arrest induced by DNA damage is associated with a down-regulation of mitotic cyclins, cdk1, and cdc25C phosphatase expression. We found that the promoter activity of these genes is repressed in the G2 arrest induced by DNA damage. We asked whether the CCAAT-binding NF-Y modulates mitotic cyclins, cdk1, and cdc25C gene transcription during this type of G2 arrest. In our experimental conditions, the integrity of the CCAAT boxes of cyclin B1, cyclin B2, and cdc25C promoters, as well as the presence of a functional NF-Y complex, is strictly required for the transcriptional inhibition of these promoters. Furthermore, a dominant-negative p53 protein, impairing doxorubicin-induced G2 arrest, prevents transcriptional down-regulation of the mitotic cyclins, cdk1, and cdc25C genes. We conclude that, as already demonstrated for cdk1, NF-Y mediates the transcriptional inhibition of the mitotic cyclins and the cdc25C genes during p53-dependent G2 arrest induced by DNA damage. These data suggest a transcriptional regulatory role of NF-Y in the G2 checkpoint after DNA damage.

In mammalian cells, progression through the G2 phase of the cell cycle is mediated by the activity of a specific set of proteins, which includes mitotic cyclins A, B1, and B2, mitotic kinase cdk1 (alias p34cdc2), and mitotic phosphatase cdc25C. The kinase activity of cdk1 during the G2 phase is dependent on its dephosphorylated status of specific residues triggered by the phosphatase activity of cdc25C protein (1–6), as well as on the levels of cyclins A and Bs. In proliferating cells, oscillations of mitotic cyclin amounts are tightly regulated at the transcriptional level (7–12). In particular, activation of cdk1 does not occur until sufficient cyclin B1 protein is synthesized (13). The accumulation of mitotic cyclins and cdk1 correlates with nascent gene expression, and their mRNAs can only be detected in particular phases of the cell cycle (2, 7, 10, 14).

Cell cycle progression through the G2 phase is controlled by the G2 checkpoint. This checkpoint ensures correct DNA synthesis during cell proliferation and genome integrity after DNA damage. In the latter condition, cells can arrest at the G1 and/or the G2 checkpoint, depending on cell type, cell cycle phase, and checkpoint integrity (15–17). The G1/G2 arrest after DNA damage is regulated, at least in part, by the activities of the tumor suppressor gene p53. Indeed, γ-irradiated cells, knockout for the p53 gene, progress from the G2 to the M phase and maintain DNA content of 4n because of cytokinesis failure (18). Overexpression of an exogenous wild type p53 induces G2 arrest associated with down-regulation of cyclin B1, cyclin A, and cdk1 expression (19, 20). In agreement with these observations, it has been reported that wild type p53 overexpression in p53-null cells can suppress the transcriptional activity of cyclin A, cyclin B1, and cdk1 promoters (19, 21–24). However, the specific molecular mechanisms responsible for the down-regulation of the transcription of these genes in the G2 arrest are still unknown.

The CCAAT motif, G/GA/ACCAATC/GA/GC/G, is present in 30% of the eukaryotic promoters of tissue-specific, housekeeping, and cell cycle-regulatory classes of genes (25). NF-Y1 has been shown to bind to more than 120 CCAAT-containing promoters (26). It is composed of three subunits, NF-YA, -B, and -C, whose highly conserved genes have been cloned in mammals, yeast, plants, and parasites (27–31). All three subunits are required for CCAAT binding (32, 33). The promoters of cyclin A, cyclin B1, cyclin B2, and cdc25C genes all contain CCAAT boxes, and it has been demonstrated that NF-Y modulates, at least in part, their activity during the cell cycle (11, 33, 34). Thus, we asked whether NF-Y is involved in the modulation of these promoter activities during the G2 arrest induced by DNA damage.

For this purpose, we induced a G2 arrest by doxorubicin (Adriamycin; ADR)-mediated DNA damage in C2C12 non-transformed skeletal muscle cells, which possess a wild type p53 (35). We found that ADR treatment down-regulates the promoter activities of the mitotic cyclins, cdk1, and cdc25C genes. This down-regulation involves a molecular mechanism requiring, at least in part, the CCAAT boxes and the transcription factor NF-Y. By using a dominant-negative p53 protein (DD-p53), we also show that the NF-Y-dependent down-regulation of mitotic cyclins, cdk1, and cdc25C occurs only in the presence of a functional p53 protein.

* This work was supported in part by Telethon-Italy Grant 1035 and the Associazione Italiana per la Ricerca sul Cancro. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipients of an Italy-United States of America project fellowship.  
¶ To whom correspondence should be addressed: Laboratorio Oncogenesi Molecolare, Istituto Regina Elena, CRS, Via delle Messi D’Oro, 156, Rome 00158, Italy. Tel.: 39-06-4985-2531; Fax 39-06-4985-2505; E-mail: piaggio@ifo.it.

§§ Recipients of an Fondazione Italiana per la Ricerca sul Cancro

‡ ‡‡ To whom correspondence should be addressed: Laboratorio Oncogenesi Molecolare, Istituto Regina Elena, CRS, Via delle Messi D’Oro, 156, Rome 00158, Italy. Tel.: 39-06-4985-2531; Fax 39-06-4985-2505; E-mail: piaggio@ifo.it.

1 The abbreviations used are: NF-Y, nuclear factor Y; ADR, Adriamycin; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; LUC, luciferase; CMV, cytomegalovirus.
NF-Y Repression of Mitotic Cyclins during the G2 Arrest

EXPERIMENTAL PROCEDURES

Cell Cycle Analysis—DNA distribution analysis of propidium iodide-stained cells was performed according to standard procedures. For each sample 105 events were analyzed by an Epics cytofluorometer (Coulter). DNA content and cell cycle distribution were determined using computer-assisted analysis.

Mitotic Index—The cells were washed twice with phosphate buffered saline (PBS) and fixed with 2% formaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.25% Triton X-100, nuclei were stained with Hoechst 33342.

Terminal Nucleotidyl Transferase Assays—Approximately 2 × 104 cells were cyto-centrifuged onto glass slides, and cytospin preparations were air dried, fixed with paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature, rehydrated with PBS, incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice (4°C), and preblocked for 30 min at room temperature. Cells were incubated with fluorescein-conjugated dUTP terminal deoxynucleotidyl transferase mixture for the assay (Roche Diagnostics S.p.A., Monza, Italy) following the manufacturer’s conditions, counterstained with 0.1 μg/ml Hoechst 33342 dye for 2 min, and mounted with coverslips in 5% glycerol in PBS. Apoptosis was quantified by determining the percentage of stained terminal nucleotidyl transferase-positive cells on a total of 400 cells.

Reporter Plasmid Constructs—p332B1CAT, p240B1CAT (10), pm-tupCCAAT, pm-downCCAAT, pmtup/downCCAAT (33), pcycA LUC (39). The cdc25C-wt/luc construct was obtained by polymerase chain reaction-amplifying the human promoter region with the primers 5′-CGA GCC CAA CAG CTT AGA GGC and 3′-CGT CCT AAG TGT TGG GCT CGC AGA, cloned into the pGL3-Basic plasmid (Promega Mannheim Germany). The cdc25C deletion mutant was created by polymerase chain reaction with the primers 5′-GGG TCA CGT CCT TCT GGT GCT GGC AGA TCA TTG-3′ and cloning the 1543 to 1 nucleotidyl transferase assay was performed as shown in Table 1, no significant amount of apoptotic cells was found after ADR treatment. Fewer than 2% of the ADR-treated C2C12 cells presented mitotic figures (Table 1). We conclude that in our experimental conditions, ADR treatment generates significant accumulation of C2C12 cells in the G2 phase of the cell cycle (19% versus 50% approximately).

Mitotic Cyclins, cdk1, and cdc25C Promoter Activities Are Down-regulated in ADR-treated C2C12 Cells—It has been reported that in cells expressing wild type p53 protein, ADR-mediated DNA damage induces G2 arrest (43). To identify experimental conditions that efficiently induce an accumulation of the cells in the G2 phase, dose-response curves to ADR were performed on C2C12 cells. These conditions were reached by a 22-h treatment with 0.5 μg/ml ADR (data not shown). The following experiments were all performed under these conditions.

The typical DNA content analysis of untreated (panel A) and ADR-treated C2C12 cells (panel B) is reported in Fig. 1. Accumulation of ADR-treated cells in G2/M phases is accompanied by a decrease in the fraction of the cells in the S phase of the cell cycle. To evaluate the amount of apoptosis, the terminal nucleotidyl transferase assay was performed. As shown in Table I, no significant amount of apoptotic cells was found after ADR treatment. Finally, to distinguish between G2 and M phases, the mitotic index was evaluated. Fewer than 2% of the ADR-treated C2C12 cells presented mitotic figures (Table 1).

RESULTS

ADR-mediated DNA Damage Induces G2 Arrest in C2C12 Cells—It has been reported that in cells expressing wild type p53 protein, ADR-mediated DNA damage induces G2 arrest (43). To identify experimental conditions that efficiently induce an accumulation of the cells in the G2 phase, dose-response curves to ADR were performed on C2C12 cells. These conditions were reached by a 22-h treatment with 0.5 μg/ml ADR (data not shown). The following experiments were all performed under these conditions.

The typical DNA content analysis of untreated (panel A) and ADR-treated C2C12 cells (panel B) is reported in Fig. 1. Accumulation of ADR-treated cells in G2/M phases is accompanied by a decrease in the fraction of the cells in the S phase of the cell cycle. To evaluate the amount of apoptosis, the terminal nucleotidyl transferase assay was performed. As shown in Table I, no significant amount of apoptotic cells was found after ADR treatment. Finally, to distinguish between G2 and M phases, the mitotic index was evaluated. Fewer than 2% of the ADR-treated C2C12 cells presented mitotic figures (Table 1). We conclude that in our experimental conditions, ADR treatment generates significant accumulation of C2C12 cells in the G2 phase of the cell cycle (19% versus 50% approximately).

Mitotic Cyclins, cdk1, and cdc25C Promoter Activities Are Down-regulated in ADR-treated C2C12 Cells—It has been demonstrated recently that during the G2 arrest induced by ADR-mediated DNA damage, the expression of mitotic cyclins and cdk1 is down-regulated (22, 43). Accordingly, we found that ADR-treated C2C12 cells express lower levels of mitotic cyclin-cdk complexes, as well as cdc25C phosphatase, both at protein and mRNA levels (data not shown). To investigate whether the down-regulation of the mitotic cyclins, cdk1, and cdc25C expression after ADR treatment involves mechanisms acting at the transcriptional level, we evaluated the promoter activities of these genes. For this purpose different promoter constructs were transfected in C2C12 cells, and promoter activities were evaluated before and after ADR treatment. Both transient and stable transfectants were produced. Similar results were ob-

FIG. 1. ADR-mediated DNA damage induces a G2 arrest in C2C12 cells. Panel A, DNA distribution analysis of C2C12 cells. Panel B, after ADR-mediated DNA damage C2C12 cells accumulate in G2/M. The percentages of cells in G1/G0, S, and G2/M phases are indicated.
tained independently of the type of transfection. The following promoter constructs were employed: (i) pcycA LUC, containing human cyclin A promoter fragment from −89 to +11 base pairs (36); (ii) p332B1CAT, containing human cyclin B1 promoter fragment from −154 to +182 base pairs; (iii) p240B1CAT, containing human cyclin B1 promoter fragment from −57 to +182 base pairs (10); (iv) B2-Luci, containing 1.1 kilobase of murine cyclin B2 promoter (34); (v) pdk1CAT, containing 2 kilobases of human cdk1 promoter (14); (vi) pdc25C-wt-luci, containing 1,433 base pairs of human cdc25C promoter. The activities of these promoters in untreated cells were referred to as 100%, and the relative activities after 22 h of ADR treatment were calculated. As shown in Fig. 2, the mitotic cyclins, the cdk1, and the cdc25C promoter activities were dramatically down-regulated after ADR treatment. In contrast, ADR treatment did not down-regulate the activity of the cyclin D1 promoter, whose activity is high in the G1 phase of the cell cycle (37), as well as the activity of Eo promoter, whose activity is tissue-specific. These results indicate that the observed down-regulation cannot be attributed to a nonspecific silencing of the transcription determined by toxicity of ADR treatment. Taken together, these data demonstrate that cyclin A, cyclin B1, cyclin B2, cdk1, and cdc25C promoter activities are down-regulated in the G2 arrest generated by ADR treatment.

Mutations of the CCAAT Boxes Impair the ADR-mediated Down-regulation of the cyclin B1, cyclin B2, and cdc25C Promoter Activities—All of the above tested promoters contain a common DNA sequence, the CCAAT box. It has been demonstrated previously that the CCAAT boxes are key sequences for the promoter activities of these genes (11, 12, 33, 34). Thus, we investigated the functional contribution of these elements to the down-regulation of the promoter activities upon ADR treatment. For this purpose, constructs with mutations of CCAAT to CTGGA in the cyclin B1 promoter, CCAAT to TTACT in the cyclin B2 promoter, and deletion of the CCAAT boxes in the cdc25C promoter were employed. In the context of p240B1CAT vector, three different CAT reporter constructs carrying single or double mutations were employed. The pmtupCCAAT construct carries a single mutation in the upstream CCAAT box, whereas the pmtdownCCAAT construct carries a single mutation in the downstream element. The pmtup/downCCAAT construct carries a mutation in both CCAAT boxes (33). We used, in the context of the cyclin B2 promoter, two luciferase reporter constructs carrying two mutated elements (Y1,2) or all (three) mutated CCAAT boxes (Y1,2,3 m) (34). We also used, in the context of the cdc25C promoter, a luciferase reporter construct carrying a deletion spanning a region from −742 to −697, relative to the ATG, containing two of the three CCAAT boxes present in this promoter (cdc25C-3742/697-luci). Mutated and wild type constructs were transfected in C2C12 cells. As shown in Fig. 3D, the mutation of all CCAAT boxes impairs the down-regulation of the cyclin B1 promoter activity occurring after the ADR treatment. In the same experimental conditions, the single mutation of the upstream (Fig. 3B) or downstream (Fig. 3C) cyclin B1 CCAAT box, partially impairs the promoter activity down-regulation. These data demonstrate that the two CCAAT boxes of the cyclin B1 promoter act in a synergistic manner.

and, although to a different extent, each CCAAT box on its own mediates the effect of the ADR treatment. In the context of the cyclin B2 promoter, the mutations of all (Fig. 4C), or only two (Fig. 4B) CCAAT boxes completely abolished the down-regulation occurring after the ADR treatment. These results demonstrate that the CCAAT boxes 1 and 2 of the cyclin B2 promoter both mediate the effect of ADR treatment. In the context of the cdc25C promoter, the deletion of two CCAAT boxes leads to about 20% of down-regulation before ADR and completely abolished the down-regulation occurring after the ADR treatment (Fig. 5). These results demonstrate that the cdc25C promoter
NF-Y Repression of Mitotic Cyclins during the G2 Arrest

Fig. 4. Mutations of the CCAAT boxes impair the ADR-mediated down-regulation of the cyclin B2 promoter activity. 10 μg of pB2-Luci vector carrying the wild type cyclin B2 promoter (panel A), or Y1,2 m vector carrying mutation in two CCAAT boxes (panel B), or Y1,2,3 m vector carrying mutation in all CCAAT boxes (panel C) was transiently cotransfected in C2C12 cells. Luciferase activities were measured in untreated (white bars) and ADR-treated cells (black bars). Values are the means ± standard deviations of four independent experiments. A schematic representation of the constructs employed is shown.

Fig. 5. Mutations of the CCAAT boxes impair the ADR-mediated down-regulation of the cdc25C promoter activity. 10 μg of pcdc25C-wt-luci vector carrying the wild type cdc25C promoter (panel A) or cdc25CΔ742/697-luci vector containing two of the three CCAAT boxes present on this promoter (panel B) was transiently cotransfected in C2C12 cells. Luciferase activities were measured in untreated (white bars) and ADR-treated cells (black bars). Values are the means ± standard deviations of four independent experiments. A schematic representation of the constructs employed is shown.

region from −742 to −697 with respect to the ATG, containing two of the three CCAAT boxes present on the promoter (11), mediates the effect of the ADR treatment. Altogether, these data show that the CCAAT boxes are key sequences in the down-regulation of the cyclin B1, cyclin B2, and cdc25C promoter activities after the ADR treatment.

Dominant-negative NF-Y Protein Abrogates the Down-regulation of the cyclin B1, cyclin B2, cdc25C, and cdk1 Promoters after the ADR Treatment—The CCAAT motif is recognized by the NF-Y transcription factor (26). To evaluate the role of the NF-Y complex on the ADR-mediated down-regulation of the cyclin B1, cyclin B2, cdc25C, and cdk1 promoters we employed a dominant-negative NF-Y vector, YA13 m29 (44). Upon transfection in mammalian cells, this vector expresses a mutant protein containing a triple amino acids substitution in the NF-YA DNA binding subdomain enabling the subunit to interact with the NF-YB/NF-YC dimer. The resulting trimer is inactive in terms of CCAAT recognition. The dominant-negative NF-Y was cotransfected with p332B1CAT, p240B1CAT, B2-Luci, pcdc25C-wt-luci, and pcdk1CAT constructs in C2C12 cells, and the promoter activities were determined in the presence or absence of ADR. As demonstrated previously (33, 34), YA13 m29 decreased the activities of all tested promoters. However, the dominant-negative YA13 m29 completely impairs the ADR-mediated down-regulation of each promoter (Fig. 6). These results indicate that a functional NF-Y complex is required for the down-regulation of cyclin B1, cyclin B2, cdc25C, and cdk1 and suggest that NF-Y has a role in the modulation of the transcription of these genes during induced G2 arrest.

C2C12 Cells Expressing Dominant-negative p53 Protein Escape the G2 Arrest Induced by ADR Treatment and Enter Mitosis—It has been reported previously that p53 is essential to sustain G2 arrest after γ-irradiation (18, 45). To assess the specific effect of p53 on cell cycle progression after ADR treatment in C2C12 cells we interfered with the activity of endogenous wild type p53 by expressing a dominant-negative p53 (DD-p53) protein. For this purpose, C2C12 myoblasts were infected with the LDDSN retrovirus carrying murine DD-p53 miniprotein and, as control, with the insertless retroviral vector, LXSN (C2C12 LXSN) (46). The dominant-negative p53 protein (DDp53) is a miniprotein consisting of the last 89 residues of murine wild type p53, including the oligodimerization domain, and lacking the DNA binding and trans-acting domains. An antagonist effect of DD-p53 toward a wild type p53 protein has been reported, at least partially the result of the formation of functionally defective, mixed oligomers between the two proteins (46). As expected, the p53 protein present in the mixed oligomers has a half-life longer than that present in the wild type oligomers, irrespective of the ADR treatment (see Fig. 8A) (46). Infected cells were maintained as polyclonal populations after G418 selection. Western blot analysis assessed the stable expression of DD-p53 protein in C2C12 cells (see Fig. 8A). Flow cytometric analysis of C2C12 LXSN cells (data not shown) indicates that the insertless retroviral vector does not influence the cell cycle distribution of these cells before and after the ADR treatment compared with the noninfected cells showed in Fig. 1. The cytofluorometric analysis of DDp53-expressing cells (C2C12 LDDSN) shows that this population does not present significant alterations in cell cycle phase distribution in normal culture conditions (compare Fig. 1A with Fig. 7A). However, after the ADR treatment the same cells lose the G1 checkpoint and accumulate mainly in the G2/M phases of the cell cycle (79%, approximately versus 21% of untreated cells) (Fig. 7B). This result indicates that DD-p53 protein, interfering with p53 activity, impairs the checkpoints of the cell cycle. To distinguish between G2 and M phases, the mitotic index was evaluated. As shown in Table II, after ADR treatment ~35% of C2C12 LDDSN cells entered mitosis and formed micronuclei. These results indicate that C2C12 cells expressing a dominant-negative p53 protein overcome the G2 checkpoint and accumulate at the end of the M phase. We, therefore, conclude that as reported previously for γ-irradiation in other cell types (18, 45) in C2C12 cells the G2 arrest generated by ADR-mediated DNA damage is p53-dependent.

Dominant-negative p53 Protein Abrogates the ADR-mediated Down-regulation of Mitotic Cyclin Complexes—It has been reported that exogenous wild type p53 overexpression down-regulates cyclin B1, cyclin A, and cdk1 expression both at transcriptional and translational levels (19, 20, 23, 24, 47). To
assess whether the down-regulation of these genes after ADR treatment in C2C12 cells requires the presence of wild type p53, Western blot analysis was performed on the C2C12 LDDSN cells and, as control, on C2C12 cells infected with the insertless retroviral vector, LXSN (C2C12 LXSN). As shown in Fig. 8A, after 22 h of ADR treatment, there is a substantial decrease of cyclin A, cyclin B1, and cdc25C proteins in these cells. Although to a lesser extent, there is also a decrease of cdk1 expression. As expected, p53 levels are increased because of a stabilization of the protein which occurs when the DD-p53 miniprotein is expressed (46). DD-p53 expression significantly reduced the ADR-mediated down-regulation of cyclin A, cyclin B1, and cdc25C. Indeed, after DNA damage, cdk1 expression is completely rescued by the presence of DD-p53, whereas 74% of cyclin A, 50% of cyclin B1 and cdc25C expression was rescued in the same conditions, as measured by densitometric analysis. We also tested, in the same experiments, the expression of cdk7 and cdk9 kinases. Both of these kinases are involved in transcriptional control through the phosphorylation of the RNA polymerase II C-terminal domain (48). As shown in Fig. 8A, after 22 h of ADR treatment, there is a substantial decrease of cdk7 protein in C2C12 LXSN cells, and DD-p53 expression significantly reduced this down-regulation. The expression of cdk9 is not affected at all by the treatment irrespective of the presence or absence of a functional p53 protein. These data confirm that mitotic cyclin-cdk complexes are modulated by wild type p53 protein upon ADR-mediated DNA damage and demonstrate also that cdc25C and cdk7 expression is modulated by wild type p53 in these experimental conditions.

Thereafter, the activity of mitotic cyclins, cdk1, and cdc25C promoters was evaluated in ADR-treated and untreated C2C12 LDDSN cells. As shown in Fig. 8B, the down-regulation of promoter activities induced by ADR treatment was inhibited substantially in C2C12 cells expressing a dominant-negative NF-Y protein (Fig. 6). Dominant-negative NF-Y abrogates the down-regulation of the cyclin B1, cyclin B2, cdc25C, and cdk1 promoters after ADR treatment. C2C12 cells were transfected with 5 μg of p332B1CAT (panel A), or p240B1CAT vectors carrying the wild type cyclin B1 promoter (panel B), or pB2-Luci vector carrying the wild type cyclin B2 promoter (panel C), or pcdc25C-wt-luci carrying the wild type cdc25C promoter (panel D), or pcdk1CAT carrying the wild type cdk1 promoter (panel E). These promoter fragments were cotransfected with 5 μg of eukaryotic vector expressing dominant-negative NF-YA protein YA13 m29. CAT and luciferase activities were measured in untreated (white bars) and ADR-treated cells (black bars). Values are the means ± standard deviations of four independent experiments.

Table II

|                  | Mitotic cells | Micronucleated cells |
|------------------|---------------|----------------------|
| C2C12 LDDSN      | 6.33%         | 0%                   |
| C2C12 LDDSN + ADR| 0.86%         | 35.06%               |

Fig. 7. C2C12 cells expressing dominant-negative p53 protein escape the G2 arrest induced by ADR treatment and enter mitosis. Panel A. DNA distribution analysis of C2C12 cells infected with the LDDSN retrovirus, carrying a dominant-negative p53 protein (C2C12 LDDSN). Panel B, after ADR-mediated DNA damage, dominant-negative p53-expressing cells escape the G2 arrest and mostly accumulate in G2/M phases. The percentages of cells in G0/G1, S, and G2/M phases are indicated.
p53 protein. The cyclins and cdc25C promoters were more sensitive to the presence of a functional p53 protein than the cdk1 promoter. Altogether these data demonstrate that the down-regulation of transcription of mitotic cyclins, cdk1, and cdc25C genes mediated by ADR-induced DNA damage depends on the presence of a functional p53 protein.

**DISCUSSION**

The progression through the G2 phase of the cell cycle is regulated in part by the cyclin A-cdk1 and cyclin B-cdk1 mitotic complexes (49, 50). Although it has been described that after DNA damage the mitotic entry is inhibited (18, 43, 51), the molecular mechanism sustaining the G2 arrest is not completely elucidated. In this report, we show that during the ADR-mediated G2 arrest, a decrease of protein expression levels of cyclin A, cyclin B1, cdk1, and cdc25C caused, at least in part, by a transcriptional level of regulation, is observed. Indeed, we demonstrate that after ADR treatment the promoter activities of the cyclin A, cyclin B1, cyclin B2, cdk1, and cdc25C genes are down-regulated. These results indicate that key molecules that control G2/M transition in the normal cell cycle are transcriptionally modulated during the G2 checkpoint induced by DNA damage.

The repression of cyclin B1, cyclin B2, and cdc25C promoters, occurring in the G2 arrest, requires the integrity of the CCAAT boxes present in the 5′ region of these genes, suggesting a role for the transcription factor NF-Y (Figs. 3 and 4). It has been demonstrated previously that NF-Y binds the CCAAT boxes of cyclin B1, cyclin B2, and cdc25C promoters (11, 33, 34). Here we show that a functional NF-Y complex is required for the inhibition of cyclin B1, cyclin B2, and cdc25C promoters during G2 arrest. Indeed, the expression of a dominant-negative NF-YA abolishes the ADR-mediated repression of these promoters. Moreover, as already demonstrated, this is true also for the cdk1 promoter (24).

The down-regulation of expression and transcription of mitotic cyclin-cdk1 complexes, as well as of cdc25C phosphatase, after DNA damage requires the presence of a functional p53 protein (Fig. 8). In agreement with this result it has been reported that overexpression of p53 in p53-null cells suppresses the transcriptional activity of cyclin A, cyclin B1, cyclin B2, and cdk1 promoters (19, 21–24, 52). Nevertheless, this is the first evidence that the cdc25C promoter activity is down-regulated also.

None of the tested promoters contains a canonical DNA binding site for p53, thus our results lead to the speculation that p53 could interfere with the function of NF-Y. Indeed, it has been described that p53 protein can repress transcription by binding to and preventing the function of specific transcription factors (53, 54). In shift experiments performed with anti-NF-Y we observed that NF-Y is present on the CCAAT boxes of cyclin B1 and cyclin B2 promoters before and after ADR-treatment. Thus, p53 does not interfere with the DNA binding of NF-Y. Another possibility is that p53 interferes with the NF-Y recruitment of coactivators and/or general transcription factors. Indeed, it has been shown that p53 and NF-Y bind to overlapping domains of the p300 coactivator (55, 56), and both p53 and NF-Y bind to TATA-binding protein (57, 58).

Interestingly, NF-Y was shown also by other groups to be required for the p53-mediated inhibition of cdk1 transcription (23, 24). Furthermore, NF-Y binds the CCAAT box contained in the cyclin A promoter (11). These findings lead to the speculation that NF-Ys modulates cdk1 and cyclin A transcription after ADR-mediated G2 arrest by the same mechanism described here for the cyclin B1, cyclin B2, and cdc25C genes.

It has been shown recently that an endogenous p53 protein sustains a G2 arrest induced by ADR through a pRb-dependent decrease of cyclin B1 and cdk1 expression (43). However, the pRb-dependent inhibition of cyclin B1 expression does not seem to be caused by direct binding of pRb/E2F family proteins to the cyclin B1 promoter. Indeed, as assessed by chromatin cross-linked immunoprecipitation, an anti-E2F1 antibody does not immunoprecipitate, from cycling cells, chromatin containing the cyclin B1 promoter, whereas an anti-NF-Y antibody does.

The transcriptional regulation of the expression of mitotic kinase complexes is not the only mechanism that sustains a G2 block. Indeed, it has been demonstrated recently that in a human colorectal cancer cell line, the p53-dependent G2 arrest after γ-irradiation is the result of induction of 14–3-3σ expression (59). Once overexpressed, 14–3-3σ blocks the cell cycle contributing to the nuclear exclusion of cdk1-cyclin B1 complexes and cdc25C phosphatase (60). Thus, after DNA damage, transcriptional down-regulation of mitotic kinase complexes (this paper) and their cytoplasmic segregation (56) might con-
comitantly sustain the G2 checkpoint.

In summary, this work provides evidences that in muscle cells, the molecular mechanism responsible for the G2 checkpoint induced by ADR-mediated DNA damage involves the ability of the NF-Y transcription factor to prevent the transcription of key regulatory molecules essential for the progression through the G2 phase of the cell cycle. This finding opens the question of whether other genes, controlled by NF-Y during the cell cycle (26), are targets of its activity in the cell cycle checkpoints.

Acknowledgments—We thank Stephen Dalton for human cdki1 promoter, Mark Wanner for cyclin B2 promoter, and Pidda Yansen-Durr for cyclin A promoter; Moshe Oren for LDDSN packaging cells; Marco Cresczenzi for helpful discussion; Giulio Tibrus for technical advice; Antonio Giordano and Antonio De Luca for anti-cdk9 antibody; and Daniela Bona for computing assistance.

REFERENCES

1. Gould, K. L., and Nurse, P. (1989) Nature 342, 39–45
2. Pines, J., and Hunter, T. (1989) Cell 58, 383–386
3. Pines, J., and Hunter, T. (1990) Nature 346, 760–763
4. Parker, L. L., and Piwnica-Worms, H. (1992) Science 257, 1955–1957
5. Mcgown, C. H., and Russell, P. (1993) EMBO J. 12, 75–85
6. Liu, F., Stanton, J. J., Wu, Z., and Piwnica-Worms, H. (1997) Mol. Cell. Biol. 17, 571–583
7. Hendeng, B., Chenivesse, X., Wang, J., Eick, D., and Brechot, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5490–5494
8. Hwang, A., Maity, A., McKenna, W. G., and Muschel, R. J. (1995) J. Biol. Chem. 270, 28419–28424
9. Lucibello, F. C., Truss, M., Zwickter, J., Ehlert, F., Beato, M., and Muller, R. (1995) EMBO J. 14, 132–142
10. Piaggio, G., Farina, A., Perrotti, D., Manni, I., Fuschi, P., Sacchi, A., and Gaetano, C. (1995) Exp. Cell Res. 216, 396–402
11. Zwickter, J., Gross, C., Lucibello, F. C., Truss, M., Ehlert, F., Engeland, K., and Muller, R. (1995) Nucleic Acids Res. 23, 3822–3830
12. Katula, K. S., Wright, K. L., Paul, H., Surman, D. R., Nuckolls, F. J., Smith, J. W., Ting, J. P., Yates, J., and Cogswell, J. P. (1997) Cell Growth Diff. 8, 11–20
13. Solomon, M. J., Glotzer, M., Lee, H., Philip, R., and Kirsh, S. (1990) Cell 63, 1013–1024
14. Dalton, S. (1992) EMBO J. 11, 797–1864
15. Kastan, M. B., Onyekwere, O., Siddranzky, D., Vogelstein, B., and Craig, R. W. (1991) Cancer Res. 51, 6304–6311
16. Maity, A., McKenna, W. G., and Muschel, R. J. (1994) Radiother. Oncol. 31, 1–13
17. Cox, L. S., and Lane, D. P. (1995) Bioessays 17, 501–508
18. Bum, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinaru, V., and Kaelin, G. (1998) Science 282, 1497–1500
19. Innocente, S. A., Abrahamson, J. L., Cogwell, J. P., and Lee, J. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2147–2152
20. Sugrue, A., Chen, Y. H., and Aaronson, S. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9648–9653
21. Yamamoto, M., Yoshida, M., Ono, K., Fujita, T., Ohtani-Fujita, N., Sakai, T., and Nakaido, T. (1994) Exp. Cell Res. 210, 94–101
22. Passama, T. M., Benati, J. A., Gewin, L., Kison, T., and Galloway, D. A. (1999) Mol. Cell. Biol. 19, 5872–5881
23. Taylor, W. R., DePrimo, S. E., Agarwal, A., Agarwal, M. L., Schonthal, A. H., Katula, K. S., and Stark, G. R. (1999) Mol. Biol. Cell 10, 3607–3622
24. Yun, J., Chae, H. D., Choy, H. E., Chung, J., Yoo, H. S., Han, M. H., and Shin, D. Y. (1999) J. Biol. Chem. 274, 29677–29682
25. Buscher, P. (1990) J. Mol. Biol. 212, 563–578
NF-Y Mediates the Transcriptional Inhibition of the cyclin B1, cyclin B2, and cdc25C Promoters upon Induced G2 Arrest

Isabella Manni, Giuseppina Mazzaro, Aymone Gurtner, Roberto Mantovani, Ulrike Haugwitz, Karen Krause, Kurt Engeland, Ada Sacchi, Silvia Soddu and Giulia Piaggio

J. Biol. Chem. 2001, 276:5570-5576.
doi: 10.1074/jbc.M006052200 originally published online November 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006052200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 26 of which can be accessed free at http://www.jbc.org/content/276/8/5570.full.html#ref-list-1