The Nuclear Death Domain Protein p84N5 Activates a G2/M Cell Cycle Checkpoint Prior to the Onset of Apoptosis*

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Programmed cell death is essential for normal development, tissue homeostasis, and host defense mechanisms. Programmed cell death is recognized by a collection of distinctive morphological and biochemical characteristics termed apoptosis (1). The pathways leading to apoptosis in response to extracellular stimuli, such as tumor necrosis factor, or in response to mitochondrial apoptotic signals, such as cytochrome c release, are well characterized (2, 3). Initiator caspases are typically recruited to protein complexes composed of death receptors and/or adapter molecules. These proteins contain signature protein interaction motifs like the death domain, the death effector domain, or the CARD domain. The locally high concentration of recruited caspase proenzyme triggers its activation by proteolytic processing thereby initiating a caspase proteolytic cascade. Apoptotic signals can also originate from within the nucleus. For example, DNA damage caused by radiation triggers a stress response that can result in apoptotic cell death (4). The mechanisms utilized by nuclear signals to initiate apoptosis are not well understood.

A number of nuclear transcription factors can induce apoptosis, including p53 (5, 6). Although p53 has a well documented role in the response of the cell to DNA damage, the mechanism used by p53 to initiate apoptosis is controversial. Although it may trigger apoptosis from within the nucleus by altering the expression of genes directly involved in the execution of apoptosis (7), non-nuclear mechanisms unrelated to transcriptional regulation have also been proposed (8). Few proteins other than transcription factors are known to require nuclear localization to initiate apoptosis. Nuclear localization of expanded polyglutamine repeat proteins is required for their ability to induce apoptosis that causes progressive neurodegenerative diseases like Huntington’s disease or spinocerebellar ataxia (9, 10). Activation of caspase-8 is a required step in this process (11). Activation of caspase-8 apparently occurs by a novel mechanism involving recruitment of the proenzyme to characteristic protein aggregates that are associated with these diseases.

Recently, we demonstrated that the nuclear protein encoded by the N5 gene (p84N5)1 was capable of initiating p53-independent apoptosis (12). Since p84N5 contains a death domain, it may participate in a nuclear apoptotic pathway. Consistent with this hypothesis, p84N5-induced apoptosis has a pattern of caspase and NF-κB activation that is similar to radiation-induced apoptosis (13). The N5 gene was originally isolated based on the ability of p84N5 to bind an amino-terminal domain of the retinoblastoma tumor suppressor protein (Rb) (14). Rb can regulate both p53-dependent and p53-independent apoptotic responses to DNA damage (15). Association with pRb inhibits p84N5-induced apoptosis, identifying p84N5 as a potential mediator of the inhibitory effects of pRb on p53-independent apoptosis. One characteristic feature of DNA damage-induced apoptosis, especially in the absence of wild-type p53, is the activation of a G2/M cell cycle checkpoint prior to cell death (16). In the current study, we test whether p84N5 activates a G2/M cell cycle checkpoint.

1 The abbreviations used are: p84N5, the protein encoded by the N5 cDNA; Rb, the retinoblastoma tumor suppressor protein; AdN5, recombinant adenovirus designed to express p84N5; AdGFP, recombinant adenovirus designed to express the green fluorescent protein; PI, propidium iodide; PBS, phosphate-buffered saline; BrdUrd, bromodeoxyuridine; ATM, ataxia telangiectasia-mutated kinase; AT, ataxia telangiectasia.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture—** SAOS-2, 293, and C-33A cell lines were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in a 5% CO2 incubator at 37 °C. The AT22JE-T cell line and the ATM-expressing derivative were cultured under the same conditions except for the addition of hygromycin B as described (17). Viable cells were counted after trypsin blue staining using a hemocytometer. Caffeine was added to the culture media to a final concentration of 2 mM.

**Plasmids and Adenovirus—** The full-length p84N5 cDNA was subcloned into the pCEP4 (Invitrogen, Carlsbad, CA) expression vector as described previously (12) to create the expression vector pCMVN5. This plasmid was used to express p84N5 upon calcium phosphate-mediated transfection. The recombinant p84N5-expressing adenovirus (AdN5) was made as described previously (13, 18) by cloning the p84N5 cDNA into the pAdCMV (AS)-BHGpa vector. The green fluorescent protein-expressing adenovirus (AdGFP) was made similarly and was provided by Dr. T. J. Liu (M. D. Anderson Cancer Center). The AdE1− adenovirus is made from the pXCLJ and pM17 plasmids and lacks adenoviral E1 but contains no foreign gene. Recombinant adenovirus was purified by CsCl equilibrium density gradient centrifugation and viral particle numbers estimated by A260 in the presence of SDS as described (19). Infectious titer was determined by an end point dilution of the temperature. After incubation with 2 N HCl for 20 min at 37 °C, 0.1 M purified by CsCl equilibrium density gradient centrifugation and viral content 2 days after infection was 13.6% compared with AdGFP-infected cells (Fig. 1C).

**Transfections and Western Blotting—** For transfections, 293 cells were seeded on 100-mm dishes and transfected the following day by overnight.

**RESULTS**

Adenoviral-mediated Expression of p84N5 Induces a G2/M Phase Cell Cycle Arrest—The full-length, wild-type N5 cDNA was used to generate a recombinant, E1-deleted, replication-defective adenovirus (AdN5) that expressed wild-type p84N5 under control of the cytomegalovirus early promoter and the bovine growth hormone polyadenylation signal (13, 18). This adenovirus was used to drive expression of p84N5 in infected cells. The cell cycle distribution of cells at different times after infection was determined by propidium iodide staining and flow cytometry. Two days after infection, the percentage of cells containing 2 N DNA content indicative of the G2/M phase increased substantially in AdN5-infected SAOS-2 and C33-A cells relative to cells infected with an adenovirus designed to express the green fluorescent protein (AdGFP) (Fig. 1A and B). AdGFP-infected SAOS-2 cells had a mean of 15.8% of cells in the G2/M phase of the cell cycle 2 days after infection, whereas AdN5-infected cells had a mean of 29.0% of cells in the G2/M phase of the cell cycle at the same time point. The mean percentage of AdGFP-infected C33-A cells with G2/M DNA content 2 days after infection was 13.6% compared with 32.8% for AdN5-infected cells. To ensure that the changes in cell cycle distribution observed were caused by expression of p84N5, similarly treated SAOS-2 cells were extracted, and the protein expression of p84N5 was analyzed by Western blotting. AdN5-infected cells show a 3–5-fold increase in accumulation of p84N5 compared with AdGFP-infected cells (Fig. 1C).

These results are consistent with induction of a G2/M cell cycle delay caused by p84N5. However, cell death or an alteration in the duration of other cell cycle phases may cause similar changes in cell cycle distribution. To confirm that p84N5 expression increases the duration of G2/M phase, we compared the kinetic parameters of the cell cycle in AdN5- and conjugated goat anti-mouse immunoglobulin G (IgG; 1:100 v/v; Sigma) in PBT and 1% normal goat serum. After a final wash in PBTB, the nuclei were resuspended in 10 μg/ml PI (Sigma) at a concentration of 106 nuclei/ml in PBTB.

Bivariate distributions of BrdUrd content (fluorescein isothiocyanate versus DNA content (PI)) were measured using an Epics 752 flow cytometer (Coulter Corp., Hialeah, FL) equipped with narrow beam (5-μm) excitation optics, a low velocity quartz flow cell and Cicer data acquisition and display electronics (Cytomation, Fort Collins, CO). Exclusion was set at 488 nm using a 5-watt argon ion laser operating at 200 miliwatts. After blocking incident laser light, BrdUrd was measured using a rhodamine fluorescent scatter and with 530-nm short pass filter and DNA content collected after a 610-nm long pass filter. There was no spectral overlap of the emitted fluorescence using this optical configuration. Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the red peak versus integral signal. Data from 30,000 events were collected in the final gated histograms. Bivariate DNA content versus BrdUrd histograms were analyzed using the “Summit software (Cytomation), and one-dimensional DNA histograms were fitted using Modfit LT (Verity Software House, Topsham, ME).

The analytical methodology for calculation of kinetic parameters has been described in detail elsewhere (21–24). The analysis is based on our established approach that we introduced to compute cell kinetic parameters using the extra information inherent in a bivariate DNA versus incorporated BrdUrd flow cytometry histogram together with our more recent method for the simultaneous estimation of the durations of the G1 and Mitosis (Tg1+M), and S phase (Ts), and the potential doubling time (Tpot) using single sample dynamic data from bivariate DNA-thymidine analogue cytometry (25). In this study labeling index (LI) was measured directly using data taken immediately (20 min) after pulse labeling and computed for the 6-h time points from LI = e−(c−v)/c−1 where c is the growth rate of the population, and v is a dimensionless quantity based on the division status of labeled cells, which may be measured from these labeled populations (22, 23, 25).

Apoptotic cells were identified by staining with Annexin-V-Alexa Flam by guest on July 25, 2018http://www.jbc.org/Downloaded from
AdGFP-infected cells. S phase cells were pulse-labeled with BrdUrd at various times after infection. Cell populations at 0 and 6 h after labeling were analyzed by bivariate flow cytometry. The histograms were used to calculate the potential doubling time, the duration of S phase, and the duration of G2/M phase for both SAOS-2 and C33-A AdN5-infected cells compared with AdGFP-infected cells (Table I). For both cell lines, the duration of G2/M phase increased approximately 2-fold. There was no consistent difference observed in the duration of S phase or the potential doubling time between AdN5- and AdGFP-infected cells.

To assess the extent of this G2/M delay, we infected cells and subsequently treated them with aphidicolin to block new DNA synthesis. Cells were delayed through G2/M, then the G2/M peak should disappear over time in the presence of aphidicolin leading to an increase in the fraction of G1 phase cells. If, however, the cells were more permanently arrested prior to mitosis, the fraction of G1 and G2/M cells would remain constant unless there is loss due to cell death. Cells infected with Ad/E1−, a nonrecombinant replication-defective adenovirus, did show a decrease in the fraction of G2/M phase cells and an increase in G1 phase cells in the presence of aphidicolin (Fig. 2).

FIG. 1. Adenovirally mediated p84N5 expression causes cells to accumulate in the G2/M phases of the cell cycle. SAOS-2 (A) or C33-A (B) cells were infected with the indicated adenovirus and then fixed and processed for PI staining and flow cytometry at the indicated times. Histograms of cell number versus PI staining intensity were generated by flow cytometric analysis of at least 10,000 cells, and the cell cycle profiles were calculated as described under “Experimental Procedures.” The shaded areas show the cell cycle profiles calculated from a representative experiment repeated at least three times. C, protein from SAOS-2 cells infected with the indicated virus was extracted 2 days after infection and analyzed for p84N5 or β-actin by Western blotting. The positions of molecular weight standards are shown at left. The position of the p84N5 and β-actin bands are shown at right.

FIG. 2. Cells G2/M arrested by AdN5 re-replicate their DNA and/or undergo apoptosis without completion of mitosis. C33-A cells were infected with the indicated virus at a multiplicity of infection of 10. Thirty two hours after infection, cells were incubated in the presence or absence of 5 μg/ml aphidicolin for an additional 16 h. Cells were then stained with propidium iodide and analyzed by flow cytometry. The data shown are the raw propidium iodide distribution with the DNA content scale compressed to show both polyploid and sub-G1 cells. The data shown are representative of three different infections for each sample.

The indicated cell lines were infected with the relevant adenovirus, and infected cells were pulse-labeled with BrdUrd either 2 (C33-A) or 4 (SAOS-2) days later. Cells were fixed immediately after pulse labeling or 6 h later and processed for bivariate flow cytometric analysis of BrdUrd and PI staining. Kinetic parameters were calculated as described under “Experimental Procedures.”

| Kinetic parameters | SAOS-2 | C33-A |
|-------------------|--------|-------|
| Labeling index (%) | 25.07  | 22.03  |
| T G1 + M (h)      | 2.50   | 5.10   |
| Tm (h)            | 56.51  | 51.98  |
| Tpot (h)          | 21.39  | 17.17  |

* a Labeling index is the percentage of BrdUrd-labeled cells at the zero time point.
* b The duration, in hours, of the G1 + M phases of the cell cycle.
* c The duration, in hours, of the S phase of the cell cycle.
* d The potential doubling time, in hours, of the cell population.
As expected, the amount of p84N5 increases 3–5-fold upon pCMVN5 transfection relative to pCMV transfection.

The G2/M Checkpoint Activated by p84N5 Expression Is Sensitive to Caffeine but Does Not Require Functional ATM Protein—Caffeine has been demonstrated to abrogate cell cycle checkpoint controls that are normally activated in response to DNA damage (26, 27). However, the type of DNA damage and the cell cycle phase in which it occurs influence whether caffeine will affect the subsequent cell cycle checkpoint (28). Abrogation of these checkpoints facilitates subsequent cell death by apoptosis. In particular, caffeine blocks G2/M cell cycle arrest and increases sensitivity to ionizing irradiation or other genotoxic treatments. The mechanisms utilized by caffeine to block activation of the G2/M cell cycle checkpoint are not completely defined. However, caffeine has been demonstrated to inhibit the ataxia telangiectasia-mutated (ATM) kinase (29, 30). ATM kinase can phosphorylate and activate the cell cycle regulator Chk2/Cds1. Activation of Chk2/Cds1, in turn, enforces a G2/M checkpoint by phosphorylating and inactivating Cdc25C. Cdc25C is normally required to remove an inhibitory phosphate on the mitotic cyclin-dependent kinase Cdk1. Loss of ATM function, therefore, compromises cell cycle checkpoints that are triggered in response to genotoxic stress (31).

We analyzed the effects of caffeine treatment on the p84N5-induced G2/M cell cycle delay and apoptosis. C33-A cells were infected with AdN5, AdGFP, or Ad/E1– in the presence or absence of caffeine. The cell cycle distribution of cells was analyzed by propidium iodide staining and flow cytometry at varying times after infection. The percentage of apoptotic cells was determined by staining with annexin-V. As expected, AdN5 infection induced a significant accumulation of cells in the G2/M phase of the cell cycle and a 2–3-fold increase in the percentage of apoptotic cells by 2 days after infection (Fig. 4). Treatment with caffeine prevented the accumulation of G2/M phase cells normally observed upon AdN5 infection (Fig. 4A). In the experiment shown, the fraction of G2/M phase cells 2 days after infection with AdN5 was 36.3% in the absence of caffeine and 19.5% in the presence of caffeine. Similar results were obtained with SAOS-2 cells (data not shown). However, caffeine treatment did not alter the percentage of apoptotic cells observed (Fig. 4B). Given the experimental conditions, it is not possible to determine whether caffeine increases the rate of apoptosis. However, the number of remaining viable cells 48 h after AdN5 infection is typically reduced by 40% in the presence of caffeine (1 × 10⁶ viable AdN5-infected cells in the absence of caffeine and 6.1 × 10⁵ viable AdN5-infected cells in the presence of caffeine), suggesting that caffeine may sensitize cells to AdN5-induced apoptotic cell death.

Since caffeine abrogated the p84N5-induced G2/M cell cycle arrest and caffeine can inhibit the ATM kinase, we tested the hypothesis that ATM may be required for activation of this checkpoint. Immortalized ataxia telangiectasia (AT) fibroblasts that lack wild-type ATM and the same cells reconstituted for ATM function by expression of recombinant ATM (AT/ATM) were infected with AdN5 or AdGFP, and the cell cycle distribution of infected cells was determined. AdGFP infection had little effect on the cell cycle distribution of these cells although a consistent small increase in the fraction of G2/M cells 2 days following infection was observed (Fig. 5). As in C33-A and SAOS-2 tumor cell lines, AdN5 caused a large increase in the percentage of cells in the G2/M phase of the cell cycle 2 days after infection. On average, the percentage of G2/M phase...
Cells were very similar.

Phase cells since the cell cycle distributions for AT and AT/ATM of BrdUrd pulse-labeled cells, the calculated duration of G2/M on analysis kinetic data obtained from bivariate flow cytometry an increase in the duration of this phase of the cell cycle. Based cells is consistent with an accumulation of cells in the G 2/M expression observed in pCMVN5-infected cells. Third, the relative increase in cyclin A and cyclin B expression observed in pCMVN5- versus pCMV-transfected cells is consistent with an accumulation of cells in the G 2/M phase of the cell cycle since the expression of these proteins peak during this phase. The accumulation of G 2/M phase cells on pCMVN5 transfection also indicates that the effects ob-
served are not dependent on adenovirally mediated gene trans-
fer. Finally, treatment of AdN5-infected cells with caffeine, a known inhibitor of the G 2/M cell cycle checkpoint, prevents accumulation of G 2/M phase cells.

DNA damage induced by ionizing radiation also activates a G 2/M checkpoint that is sensitive to caffeine. Although caffeine abrogates radiation-induced G 2/M cell cycle arrest, it sensitizes cells to radiation-induced cell death (32). This indicates that G 2/M cell cycle arrest is not a prerequisite for subsequent apoptosis. Similarly, caffeine abrogates p84N5-induced G 2/M checkpoint activation but not apoptosis. The percentage of apop-
totic AdN5-infected cells is similar in the presence or absence of caffeine. Although the percentage of apoptotic cells at the 48-h time point is not significantly altered by caffeine, caffeine does reduce the total number of viable AdN5-infected cells. These observations suggest that, like radiation, caffeine sensitizes cells to the effects of AdN5. The G 2/M arrest induced by DNA damage is persistent. In the absence of normal G 1 and S phase checkpoints, such as in Rb or p53 mutant cells, G 2/M cells arrested by DNA damage will initiate another round of DNA synthesis without completing the intervening mitosis. Polyploid cells are also observed after infection with AdN5 but not control virus Ad/E1−. Like DNA damage-induced checkpoint activation, this suggests that AdN5-infected cells ar-
rested at G 2/M can reinitiate DNA synthesis without complet-
ing the intervening mitosis. Blocking DNA synthesis by treatment with aphidicolin does not cause an increase in the percentage of AdN5 cells in G 1 phase as is observed in cells infected with Ad/E1−, but rather causes an increase in the fraction of cells with S phase DNA content. Since new DNA synthesis is blocked by aphidicolin, the increased fraction of apparent S phase cells is likely due to loss of DNA content during apoptotic cell death of G 2/M or polyploid cells. Hence, it appears as if cells arrested at G 2/M by AdN5 either re-initiate DNA synthesis and/or die by apoptotic cell death before complet-
ing mitosis and reentering G 1 phase.

Caffeine can inhibit ATM protein kinase activity and sub-
sequent activation of Chk2/Cds1 (29, 30). However, AdN5- and radiat-
ion-induced (data not shown) G 2/M cell cycle arrest still occurs in the absence of ATM, indicating that ATM-inde-
pendent mechanisms must exist to enforce a G 2/M checkpoint. This finding is consistent with the observations that Chk2 is dispen-
sable for initiation of the G 2/M phase checkpoint (35) and that ATM-independent mechanisms may exist to regulate Chk2 (28). How p84N5 expression leads to maintenance of Cdk1 in the inactive state required to prevent mitotic entry is unclear. One possibility is that forced p84N5 expression stresses the cell in a manner that induces an ATM-independent response anal-
ogous to that observed in response to DNA damage.

DNA damage is one apoptotic signal that unambiguously originates from within the nucleus. The nuclear localized Rb protein negatively regulates DNA damage-induced apoptosis (36, 37). How Rb influences DNA damage-induced apoptosis is not completely understood. The observation that an amino-
terminal domain of Rb that is dispensable for cell cycle regu-
lation may be required to inhibit some forms of apoptosis (38) suggests that the ability of Rb to regulate apoptosis may be a novel function. The amino-terminal domain of Rb is also re-
quired to bind p84N5, and this binding inhibits p84N5-induced apoptosis (12) and G 2/M arrest.2 We hypothesize that p84N5 plays a role in a cellular stress response that is similar to that triggered by radiation-induced DNA damage and, therefore, that it is a good candidate for mediating the effects of Rb on this response.

2 B. S. Poe and D.W. Goodrich, unpublished observations.
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