Nuclear Localization of Cyclin B1 Controls Mitotic Entry After DNA Damage

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Abstract. Mitosis in human cells is initiated by the protein kinase Cdc2-cyclin B1, which is activated at the end of G2 by dephosphorylation of two inhibitory residues, Thr14 and Tyr15. The G2 arrest that occurs after DNA damage is due in part to stabilization of phosphorylation at these sites. We explored the possibility that entry into mitosis is also regulated by the subcellular location of Cdc2-cyclin B1, which is suddenly imported into the nucleus at the end of G2. We measured the timing of mitosis in HeLa cells expressing a constitutively nuclear cyclin B1 mutant. Parallel studies were performed with cells expressing Cdc2AF, a Cdc2 mutant that can-

not be phosphorylated at inhibitory sites. Whereas nuclear cyclin B1 and Cdc2AF each had little effect under normal growth conditions, together they induced a striking premature mitotic phenotype. Nuclear targeting of cyclin B1 was particularly effective in cells arrested in G2 by DNA damage, where it greatly reduced the damage-induced G2 arrest. Expression of nuclear cyclin B1 and Cdc2AF also resulted in significant defects in the exit from mitosis. Thus, nuclear targeting of cyclin B1 and dephosphorylation of Cdc2 both contribute to the control of mitotic entry and exit in human cells.

Initiation of mitosis in human cells is triggered by abrupt activation of the cyclin-dependent protein kinase Cdc2 (or Cdk1; for reviews see Nurse, 1990; Dunphy, 1994; King et al., 1994; Lew and Kornbluth, 1996; Morgan, 1997). Activation of Cdc2 is a complex process that begins when the Cdc2 catalytic subunit associates with its regulatory subunit, cyclin B1, whose levels rise during S phase and G2, and peak in mitosis. Before mitosis, the expanding population of Cdc2-cyclin B1 complexes is held in an inactive state by Cdc2 phosphorylation at Thr14 and Tyr15. These phosphorylations are catalyzed by Wee1, Myt1, and related protein kinases; dephosphorylation is catalyzed by the phosphatase Cdc25C.

At the end of G2, sudden dephosphorylation of inhibitory sites on Cdc2 leads to complete activation of the Cdc2-cyclin B1 stockpile. Rapid dephosphorylation of Cdc2 is thought to result from simultaneous inactivation of Wee1-like kinases and activation of the phosphatase Cdc25C. Mitotic changes in Wee1 and Cdc25C activity are due in part to their phosphorylation by Cdc2-cyclin B1, suggesting that positive feedback loops help generate the rapid activation of Cdc2-cyclin B1 complexes (for reviews see Dunphy, 1994; Lew and Kornbluth, 1996; Morgan, 1997). Additional mechanisms also control Cdc2 dephosphoryla-

tion; for example, the protein kinase Plx phosphorylates Cdc25C in mitosis (Kumagai and Dunphy, 1996), providing a mechanism by which other regulatory systems may influence the onset of mitosis.

Although dephosphorylation of inhibitory sites in Cdc2 is clearly a key determinant of mitotic timing in the fission yeast *Schizosaccharomyces pombe* (Gould and Nurse, 1989) and in the early *Drosophila* embryo (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990), the onset of mitosis in mammalian cells is not controlled by this mechanism alone. This issue has been addressed primarily by studies of mitotic timing in mammalian cells expressing Cdc2AF, a dominant mutant form of Cdc2 in which Thr14 and Tyr15 are changed to alanine and phenylalanine, respectively. The Cdc2AF protein induces limited premature mitotic events when transiently overexpressed in mammalian cells (Krek and Nigg, 1991; Heald et al., 1993). The expression of Cdc2AF at normal levels using a tetracyclin-repressible promoter in stable HeLa cell lines has only minor effects on the timing of mitosis (Jin et al., 1996), although long-term expression of Cdc2AF decreases cell viability (Jin et al., 1996; Blasina et al., 1997). Thus, Cdc2 dephosphorylation alone is insufficient to trigger mitosis in mammalian cells.

Similar evidence has been obtained in studies of mitotic timing after DNA damage. Cell cycle checkpoint mechanisms delay mitosis when a cell suffers DNA damage in S phase or G2 (Elledge, 1996; Lydall and Weinert, 1996),

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and there is clear evidence that this delay is caused by the stabilization of inhibitory Cdc2 phosphorylation in *S. pombe* and *Aspergillus nidulans* (Furnari et al., 1997; Rhind et al., 1997; Ye et al., 1997). In mammalian cells, Cdc2 is phosphorylated at the inhibitory sites after DNA damage (Hofmann et al., 1994; Kharbanda et al., 1994; O'Connor et al., 1994; Herzinger et al., 1995; Paules et al., 1995; Poon et al., 1996), and expression of Cdc2AF reduces the damage-induced arrest in HeLa cells (Jin et al., 1996) and increases radiation sensitivity (Blasina et al., 1997), indicating that inhibitory phosphorylation helps to delay mitosis in these cells. Recent studies suggest that DNA damage in human cells leads to activation of a protein kinase, Chk1, that phosphorylates and inhibits the function of Cdc25C (Peng et al., 1997; Sanchez et al., 1997; Weinert, 1997). However, this mechanism does not account fully for the G2 arrest that occurs after DNA damage. Damaged cells expressing Cdc2AF exhibit a reduced but still considerable G2 arrest. Most importantly, Cdc2AF-expressing cells delayed in G2 by DNA damage contain near-maximal cyclin B1 concentrations and high levels of Cdc2-cyclin B1-associated kinase activity (Jin et al., 1996). Additional mechanisms must restrain mitosis in these cells despite the presence of active Cdc2-cyclin B1 complexes.

What other mechanisms might control mitotic entry? One possibility is supported by evidence from human cells and starfish oocytes that the initiation of mitosis is accompanied not just by Cdc2 dephosphorylation, but by changes in the subcellular location of cyclin B1 (Pines and Hunter, 1991; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992; Gallant et al., 1995). During S phase and G2, cyclin B1 accumulates in the cytoplasm. Just before mitosis, the majority of the cyclin B1-Cdc2 complexes are suddenly found in the nucleus, remaining there until nuclear envelope breakdown at prometaphase. As many of the key substrates for Cdc2-cyclin B1 are in the nucleus, its exclusion from this organelle may provide a mechanism to limit its effects before mitosis. Indeed, Heald et al. (1993) engineered cells containing abundant quantities of dephosphorylated active Cdc2 whose activity was restricted to the cytoplasm, and found that these active complexes did not induce premature mitotic events. Similarly, Li et al. (1997) showed that the mitosis-promoting activity of cyclin B1 in frog eggs is abolished by mutations in cyclin B1 that prevent its localization to the nucleus. Nuclear translocation of cyclin B1 is therefore positioned to serve as an additional regulatory step in the control of mitotic initiation.

The effects of Cdc2-cyclin B1 are reversed after chromosome segregation in anaphase. Thus, the inactivation of Cdc2-cyclin B1 (presumably leading to dephosphorylation of its substrates) is required for cells to exit mitosis. Inactivation of Cdc2-cyclin B1 is generally thought to occur by ubiquitin-dependent proteolysis of cyclin B1 (for review see King et al., 1996). Prevention of this destruction, for example, by expression of a nondegradable mutant cyclin, results in arrest after anaphase in cells from a variety of organisms, including mammals (Murray et al., 1989; Gallant and Nigg, 1992; Holloway et al., 1993; Surana et al., 1993; Rimmington et al., 1994; Sigrist et al., 1995; Yamano et al., 1996; Wheatley et al., 1997).

In our previous work, we speculated that DNA damage

in human cells might act both by stabilizing inhibitory phosphorylation of Cdc2 and by maintaining the cytoplasmic localization of cyclin B1 (Jin et al., 1996). This hypothesis is supported by observations that cyclin B1 is cytoplasmic in cells arrested in G2 by DNA damage (Smeets et al., 1994; P. Jin, unpublished results). In the present work we tested this hypothesis by assessing the timing of mitosis in cells engineered to express a mutant version of cyclin B1 that is targeted to the nucleus at all cell cycle stages. We find that nuclear cyclin B1 partially overrides the G2 arrest that occurs after DNA damage, suggesting that cyclin B1 localization is a rate-limiting step in the initiation of mitosis under these conditions. Our results also provide unexpected insights into the role of cyclin B1 localization and degradation in the exit from mitosis.

Materials and Methods

Cell Culture

HeLa S3 cells were maintained in DME plus 10% FCS and synchronized at G1/S with a double thymidine block protocol. Cells were plated at 0.5×10^6 cells per 100-mm dish. After 1 d, cells were arrested in S phase by treatment with thymidine (2 mM) for 17 h, and were then released from the arrest by one wash in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, pH 7.3) and refeeding with thymidine-free medium. After 9 h, cells were subjected to a second thymidine treatment. After 14 h, cells were washed once in PBS to release from the G1/S block. For ionizing radiation experiments, cells were x-irradiated at room temperature 1 h after release from G1/S arrest with a 150 kV x-ray machine (Philips Electron Optics, Mahwah, NJ) at a rate of 2.5 Gray/min for a total dose of 6 Gray. Dosage was somewhat variable in different experiments, resulting in variations in the length of the G2 arrest.

To analyze DNA content by flow cytometry, cells were trypsinized, fixed in 70% ethanol, resuspended in PBS containing RNaseA (100 μg/ml) and propidium iodide (10 μg/ml), and analyzed by a FACScan (Becton Dickinson, Mountain View, CA) and Cellquest software. To visualize condensed chromatin, ethanol-fixed cells stained with propidium iodide were placed on glass slides and examined with a fluorescence microscope (Nikon Inc., Melville, NY).

Recombinant Adenovirus Construction

Four repeats of a Myc epitope tag sequence (EQKLISEEDLN), preceded by a methionine start codon and an upstream Kozak sequence (GCCGC-CACC), were fused in frame to coding sequences of the human cyclin B1 cDNA using the NcoI site at the start codon. To construct nuclear cyclin B1, a synthetic double-stranded DNA fragment (5′-CAT GGC AAC CCC GCC GAA AAA AAA ACG CAA AGT GGA AGA TCC-3′) encoding the nuclear localization signal of SV40 Large T antigen was inserted in frame at the NcoI site between the Myc tag and cyclin B1 coding sequence. Fusion of the Cdc2AF mutant cDNA to COOH-terminal hemagglutinin (HA)[1] epitope tag sequences has been described (Jin et al., 1996).

Recombinant adenoviruses encoding Myc-cyclin B1, Myc-NLS-cyclin B1, and Cdc2AF-HA were constructed by a recently described method (Hardy et al., 1997). Each mutant cDNA was inserted downstream of a tet operator and minimal CMV promoter (from pUHD10-3; Gossen and Bujard, 1992) in a shuttle plasmid (pAdlox/Tet) carrying the 5′ sequences of the adenoviral genome, including the Ψ packaging signal but not the E1 genes; a single loxP recombination site is found 3′ of these sequences. Shuttle plasmids were cotransfected into CRE8 cells (expressing Cre recombinase) with mutant adenovirus DNA (Ψ5) in which the packaging signal Ψ at the 5′ end of the viral genome is flanked on both sides by loxP

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1. *Abbreviations used in this paper*: CDK, cyclin-dependent kinase; CRS, cytoplasmic retention signal; HA, hemagglutinin; NLS, nuclear localization signal; MOI, multiplicity of infection; PCC, premature chromatin condensation; tTA, tetracyclin transactivator.

recombination sites; in addition, the E1 and E3 genes are deleted. Cre recombinase in the CRE8 cells catalyzes loss of the packaging signal from Ψ5 viral DNA, as well as recombination of shuttle plasmid viral sequences with the Ψ-deleted Ψ5 viral DNA, resulting in replacement of the left end of the Ψ5 DNA with the 5′ viral sequences from the shuttle plasmid. The product is a viral genome that carries a normal packaging signal as well as the foreign cDNA under Tet control. Recombinant virus is packaged and released from the cell, but is replication-defective because E1 genes are deleted (Hardy et al., 1997). A recombinant adenovirus expressing the tetracyclin transactivator (tTA) was constructed with the tTA cDNA and upstream CMV promoter from pUHD15-1 (Gossen and Bujard, 1992).

Recombinant adenoviruses were plaque-purified, amplified, and titered in 293 cells. 4 h before release from the G1/S arrest, HeLa cells were infected with the tTA virus plus the desired cyclin B1 or Cdc2AF virus or viruses, each at a multiplicity of infection (MOI) of 5–10 plaque-forming units per cell. Secondary immunofluorescence with antibodies against the Myc epitope tag revealed that 90–95% of cells in the infected population express the foreign protein in these experiments.

Immunofluorescence Analysis

For α-Myc analysis, cells growing on coverslips were rinsed with PBS, fixed with methanol/acetone (1:1) for 5 min, and incubated for 30 min at room temperature with α-Myc monoclonal antibody (9E10 ascites fluid, diluted 1:250). For immunostaining with α-lamin A and MPM-2, cells growing on cell culture dishes were collected by trypsinization and fixed in 3% formaldehyde/2% sucrose in PBS for 20 min. Fixed cells were centrifuged onto coverslips, washed with PBS, and incubated for 5 min with PBS containing 0.5% Triton X-100. Incubations with primary antibodies, diluted at 1:1000, were carried out for 30 min at room temperature. Mouse monoclonal antibody against lamin A was provided by Erich Nigg (University of Geneva); MPM-2 monoclonal antibody was provided by Arshad Desai (University of California, San Francisco). After five washes with PBS, FITC-conjugated secondary antibodies were added for 30 min. Cells were washed with PBS, incubated for 5 min with Hoechst 33258 (50 ng/ml), mounted in 90% glycerol containing o-phenyldiamine, and examined with a fluorescence microscope (Nikon Inc., Melville, NY).

Biochemical Procedures

To prepare cell lysates, cells were detached from dishes with a rubber policeman, harvested by centrifugation, and resuspended in lysis buffer (10 mM Hepes-NaOH, pH 7.4, 10 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 80 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM phenyl methylsulfonyl fluoride, 1 μg/ml leupeptin, 20 U/ml aprotinin). Lysates were clarified by centrifugation (10,000 g for 10 min at 4°C). To measure histone H1 kinase activity, cell lysate (25 μg) was incubated for 1 h at 4°C with protein A-Sepharose and affinity-purified rabbit anti-cyclin B1 antibody (raised against baculovirus-derived and purified human cyclin B1 protein; a gift of Catherine Takizawa (University of California, San Francisco). Immunoprecipitates were washed three times with HBS (10 mM Hepes-NaOH, pH 7.4, 150 mM NaCl) containing 0.1% Triton X-100, twice with HBS containing 1 mM DTT, and incubated in HBS (30 μl) containing 10 mM $MgCl_2$, 50 μM ATP, 1 μCi [γ-^{32}P]ATP (3,000 mCi/mmol; Amersham Corp., Arlington Heights, IL), 1 mM DTT, and 5 μg of histone H1 (Boehringer Mannheim Corp., Indianapolis, IN). After 5 min at 24°C, reaction products were analyzed by SDS-PAGE and autoradiography. Immunoblotting was performed with ECL Western blotting detection reagents (Nycomed Amersham, Buckinghamshire, UK).

Results

Expression of Foreign Cyclin Proteins in HeLa Cells with Recombinant Adenoviruses

To explore the role of cyclin B1 localization in mitotic timing, we used the straightforward approach of analyzing the biological effects of forcing cyclin B1 into the nucleus before mitosis. We fused a nuclear localization signal (NLS) from SV40 large T antigen to the amino terminus of human cyclin B1; previous studies indicate that attachment of an NLS causes constitutive nuclear localization of cyclin

B1 (Pines and Hunter, 1994). A quadruple Myc-epitope tag was also added to the amino terminus of the NLS-tagged cyclin B1; a Myc-tagged wild-type cyclin B1 was constructed for control experiments. This epitope tag allowed us to assess the expression of foreign cyclin proteins, and distinguish them from endogenous cyclin B1. The amino-terminal Myc and NLS tags do not interfere with the ability of cyclin B1 to bind and activate Cdc2 (data not shown).

Recombinant adenovirus vectors were used to transiently express foreign proteins in HeLa cells. We used a recently developed method (Hardy et al., 1997; see Materials and Methods) to construct replication-defective recombinant adenoviruses in which the desired cDNA is under the control of a tetracyclin-repressible promoter; expression from this promoter requires that cells are coinfected with a recombinant adenovirus encoding the tTA transactivator (Gossen and Bujard, 1992). Infection with these recombinant viruses provides a rapid method to induce expression of foreign genes in almost 100% of the cell population. Protein expression can be tightly controlled in this system by varying the MOI. We used an MOI of 5–10 pfu/cell, resulting in moderate levels of cyclin B1 expression that were about three to fivefold higher than normal mitotic levels.

Recombinant adenoviruses lack the E1 genes, the major source of viral proteins known to affect the cell division cycle (Shenk, 1996). In addition, the remaining early gene products that affect cell division (products of the E4 genes) are not expressed until over 24 h after infection (Hardy et al., 1989). As a result, infection with control viruses, such as the virus encoding the tTA protein, does not result in detectable effects on cell cycle progression during the 24-h time course of our experiments (data not shown).

Nuclear Cyclin Import Collaborates with Cdc2 Dephosphorylation to Control Mitotic Entry

We first established the effects of NLS-tagged cyclin B1 on the timing of mitosis under normal growth conditions. HeLa cells were synchronized at the beginning of S phase with a double thymidine block, infected with recombinant adenoviruses for 3–4 h, and then released from the arrest. Immunofluorescence analysis of Myc-tagged cyclin B1 in these cells confirmed that cyclin B1 was located predominantly in the cytoplasm during S phase and G2, while the NLS-tagged cyclin B1 was found entirely in the nucleus (Fig. 1 A).

In control cells infected only with the tTA virus, mitosis occurred ∼8–12 h after release from the G1/S block, as indicated by flow cytometric analysis of DNA content (Fig. 1 C, a) and analysis of chromosome condensation by fluorescence microscopy of cells stained with propidium iodide (Fig. 1 D). Uninfected HeLa cells displayed essentially identical behavior (data not shown). Biochemical analysis of lysates of control cells (Fig. 1 B, a) confirmed that cyclin B1 protein and associated kinase activity peaked at 8 h. Immunoblotting of Cdc2 revealed that a fraction of Cdc2 exhibited the decreased gel mobility that is known to be caused by phosphorylation of Thr14 and Tyr15 (Norbury et al., 1991; Solomon et al., 1992); this shift disappeared as cells progressed through mitosis.
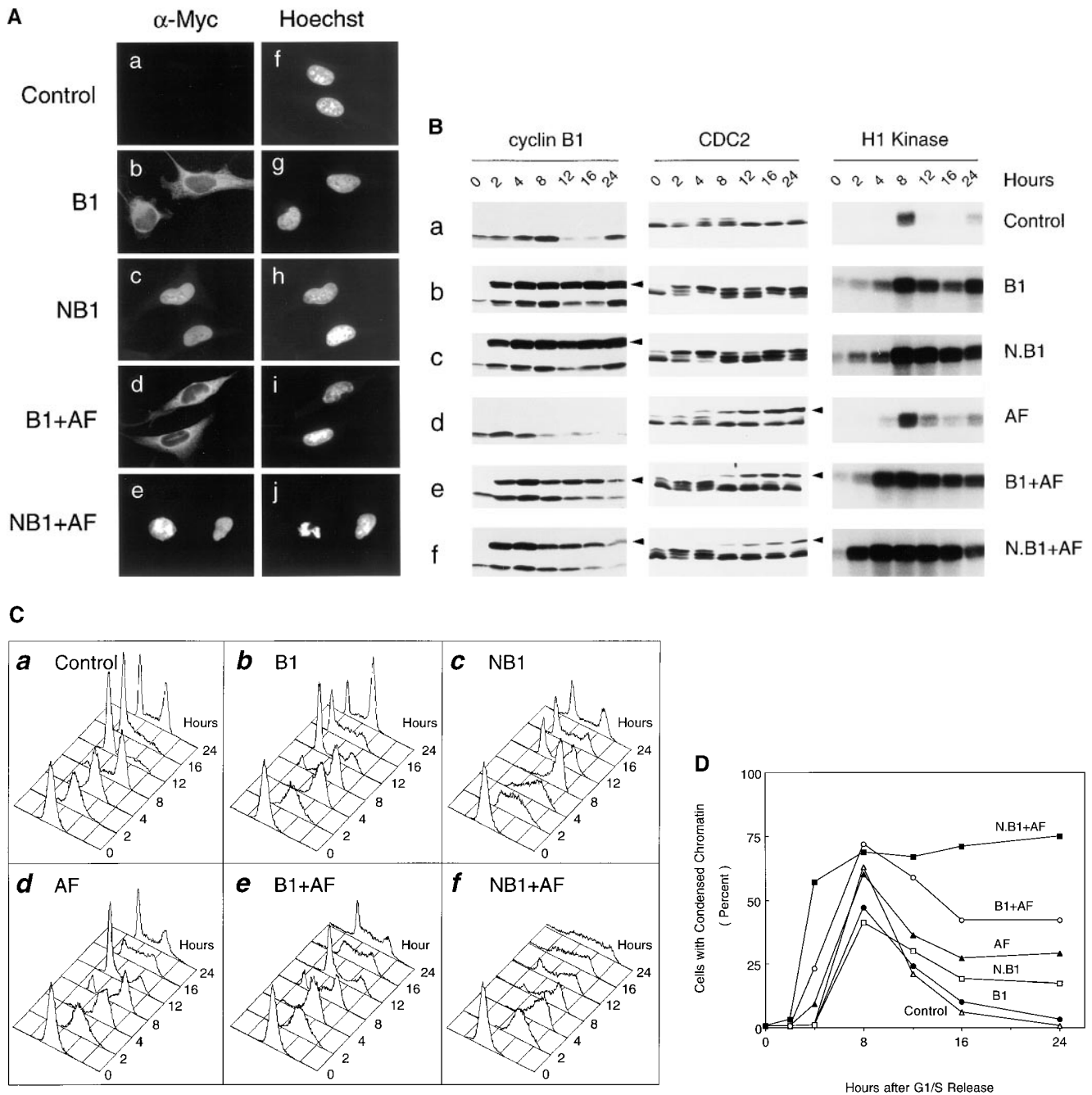
Figure 1. Effects of nuclear cyclin B1 and Cdc2AF on progression through mitosis. (*A*) HeLa cells were synchronized at the G1/S boundary by a double thymidine treatment and infected for 3 h with recombinant adenoviruses encoding the tTA transactivator (*a* and *f*, control) or with multiple viruses encoding tTA plus cyclin B1 (*b* and *g*, B1), NLS-cyclin B1 (*c* and *h*, NB1), both cyclin B1 and Cdc2AF (*d* and *i*, B1 + AF), or both NLS-cyclin B1 and Cdc2AF (*e* and *j*; NB1 + AF). 4 h after release from G1/S arrest, cells were subjected to secondary immunofluorescence analysis with an antibody against the Myc epitope tag on cyclin B1 (α-Myc; *a–e*) and treated with Hoechst 33258 to label nuclear DNA (*f–j*). (*B*) HeLa cells were synchronized at the G1/S boundary and infected for 4 h with recombinant adenoviruses encoding the tTA transactivator (*a*, Control) or with multiple viruses encoding tTA plus cyclin B1 (*b*, B1), NLS-cyclin B1 (*c*, N.B1), Cdc2AF (*d*, AF), both cyclin B1 and Cdc2AF (*e*, B1 + AF), or both NLS-cyclin B1 and Cdc2AF (*f*, N.B1 + AF). Cell lysates prepared at the indicated times after G1/S release were subjected to immunoblotting with antibodies against cyclin B1 (*left*), or antibodies against the PSTAIRE sequence conserved among CDKs (*middle*; Cdc2 is the major anti-PSTAIRE antigen in HeLa cells). Arrowheads indicate the epitope-tagged, virally-encoded cyclin B1, NLS-cyclin B1, and Cdc2AF proteins. Histone H1 kinase activity was measured in immunoprecipitates with anti-cyclin B1 (*right*); autoradiographic exposure times were 1.5 h. (*C*) Cells from the same experiment as in *B* were harvested at the indicated times, fixed, stained with propidium iodide, and analyzed by flow cytometry to measure DNA content. Abbreviations are as given in *B*. (*D*) A fraction of the cells prepared for flow cytometric analysis in *C* were examined by microscopy for the presence of condensed chromosomes. At least 300 cells were analyzed for each sample. These values represent the means of data obtained from two separate experiments, in which results were essentially identical. Abbreviations are as given in *B*.

Expression of Cdc2AF, tagged at its COOH terminus with an HA epitope tag, induced moderate effects similar to those observed in our previous studies (Jin et al., 1996): low levels of premature chromosome condensation (PCC) were observed 4 h after release from G1/S (Fig. 1 *D*), and DNA content analysis revealed a small fraction of cells that progressed prematurely into G1 (Fig. 1 *C*, *d*). Cdc2 dephosphorylation and cyclin degradation also occurred slightly earlier than in control cells (Fig. 1 *B*, *d*).

Expression of cyclin B1 or NLS-tagged cyclin B1 had no significant effects on the timing of mitotic entry (mitotic exit will be discussed below). In both cases, PCC was not observed, and mitosis in the majority of cells occurred at about the same time as in control cells (Fig. 1, *C* and *D*). Cyclin-associated kinase activity was higher in these cells than in control cells, but still rose to a peak at the 8-h time point (Fig. 1 *B*). Inhibitory phosphorylation of Cdc2 was also increased, presumably due to increased cyclin levels (Cdc2 phosphorylation is cyclin-dependent; Solomon et al., 1990). We conclude that nuclear localization of cyclin B1 alone is insufficient to trigger premature mitotic events under these conditions.

We speculated that nuclear cyclin B1 might be prevented from inducing mitotic events because the Cdc2–cyclin B1 complex is phosphorylated by Wee1 in the nucleus, as suggested by previous work (Heald et al., 1993). Consistent with this hypothesis, we found that coexpression of nuclear cyclin B1 and Cdc2AF induced PCC in over half of the population only 4 h after release from the G1/S block (Fig. 1 *D*). These effects are particularly noteworthy because the level of Cdc2AF protein expressed at early time points in these experiments was very low; coinfection tended to reduce expression of both proteins (Fig. 1 *B*, *f*). Coexpression of Cdc2AF and wild-type cyclin B1 had moderate effects on mitotic timing: PCC occurred in about 25% of cells 4 h after G1/S release (Fig. 1 *D*).

Premature mitotic events in coinfected cells were accompanied by premature activation of Cdc2-cyclin B1 complexes (Fig. 1 *B*; compare H1 kinase activities in *a* and *f* at the 2- or 4-h time points). The premature activation of Cdc2 by nuclear cyclin B1 in cells expressing barely detectable Cdc2AF suggests that the association of nuclear cyclin B1 with a very low level of Cdc2AF can trigger the complex process leading to Cdc2 dephosphorylation.

Mechanisms Controlling Exit from Mitosis

Although our original goal in this work was to explore mechanisms controlling entry into mitosis, our results also provide new insights into mechanisms controlling mitotic exit. In particular, we were surprised to find that cells expressing cyclin B1 or NLS-tagged cyclin B1 appeared to be exiting mitosis (Fig. 1, *C* and *D*) despite the fact that the exogenous Myc-tagged cyclin B1 in these cells was only partially degraded (Fig. 1 *B*, *b* and *c*). At first glance, this result does not appear consistent with the abundant previous evidence indicating that cyclin B1 destruction is required for exit from mitosis.

To explore the mechanisms of mitotic exit in cells expressing cyclin B1, we first asked why the exogenous cyclin B1 was not completely destroyed in late mitosis. Myc-tagged cyclin B1 proteins were expressed at high levels in insect cells with recombinant baculoviruses, and were added to mitotic (cytostatic factor–arrested) extracts of *Xenopus laevis* eggs that were then induced to exit from mitosis. We found that the half-lives of Myc- and Myc/NLS-tagged cyclin B1 proteins were both about 10-fold greater than the half-life of untagged human cyclin B1, although both were significantly less stable than a mutant cyclin B1 lacking its ubiquitin-targeting destruction box sequences (data not shown). Thus, the amino-terminal Myc epitope tag interferes partially with mitotic cyclin destruction.

How are cells exiting mitosis in the presence of abundant quantities of cyclin B1? One potential explanation was provided by immunoblotting analysis of endogenous Cdc2 in these experiments. Whereas Cdc2 was fully dephosphorylated in control cells passing through mitosis (Fig. 1 *B*, *a*), a major fraction of Cdc2 remained phosphorylated in cells exiting mitosis in the presence of exogenous cyclin B1 (Fig. 1 *B*, *b* and *c*). This evidence suggests that inhibitory Cdc2 phosphorylation contributes to the inactivation of Cdc2 in these cells (however, Cdc2 phosphorylation cannot be the sole mechanism allowing exit from mitosis, as Cdc2 activity declines only partially after mitosis, as discussed below).

If inhibitory phosphorylation helps to inactivate Cdc2 in cells expressing cyclin B1, then cells coexpressing cyclin B1 and Cdc2AF should exhibit defects in the exit from mitosis. Results from our coinfection experiments are consistent with this possibility. Analysis of chromosome condensation in cells coexpressing cyclin B1 and Cdc2AF indicated that a significant fraction of these cells (generally 40–50% in different experiments) did not exit mitosis, but remained in a mitotic state with condensed chromosomes (Figs. 1 *D* and Fig. 2). Cells in this prolonged mitosis did not contain intact nuclear envelopes, and contained abundant quantities of MPM-2 antigens, which are generally thought to represent mitotic phosphoproteins (Fig. 2). Thus, a large fraction of coinfected cells were arrested in a mitotic state, which is consistent with the notion that mitotic exit is defective when both cyclin degradation and inhibitory phosphorylation are reduced.

Chromosomes in coinfected cells displayed the abnormal clumped morphology that occurs when partial condensation occurs at inappropriate cell cycle stages (Fig. 2). This morphology is distinct from the chromatin compaction that occurs in cells undergoing apoptosis. We measured the extent of programmed cell death in cells coexpressing Cdc2AF and cyclin B1 by immunocytochemical detection of the abundant DNA ends that are generated during apoptosis. Apoptotic nuclei were observed in ∼10% of cells 24 h after coinfection, when 50% of cells exhibited PCC; at 48 h after infection, when all cells displayed PCC, ∼35% of cells were apoptotic (data not shown). We therefore conclude that programmed cell death eventually follows and is perhaps a consequence of premature mitotic events or mitotic arrest.

It seems unlikely that the mitotic arrest we observe in coinfected cells is due to activation of the spindle assembly checkpoint (Elledge, 1996; Rudner and Murray, 1996), which might be expected to arrest cells in metaphase in response to defects in the attachment of abnormally condensed chromosomes to the mitotic spindle. Unlike the mitotic arrest that accompanies spindle damage, the mi-
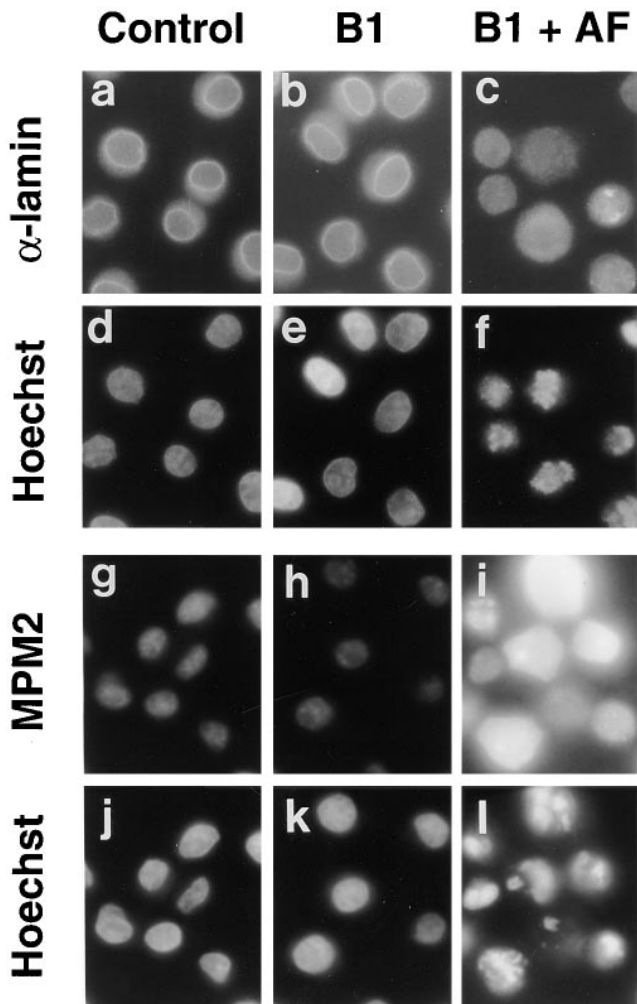
Figure 2. Cells coexpressing cyclin B1 and Cdc2AF arrest with a mitotic phenotype. HeLa cells arrested at the G1/S boundary were infected with tTA virus alone (*left*, control), tTA virus plus cyclin B1-expressing adenovirus (*middle*, B1), or tTA virus plus both cyclin B1- and Cdc2AF-expressing viruses (*right*, B1 + AF). 16 h after G1/S release, when control cells had entered the next G1 (see Fig. 1), cells were subjected to secondary immunofluorescence analysis with antibody against nuclear lamin A (*a–c*) or the MPM-2 antibody, which recognizes mitotic phosphoepitopes (*g–i*). The same cells were also stained with Hoechst 33258 to reveal chromosome morphology (*d–f* and *j–l*). At this time point, ~50% of cells coexpressing cyclin B1 and Cdc2AF display the mitotic phenotype shown in the right panels (see Fig. 1).

totic arrest in our experiments is accompanied by degradation of the endogenous cyclin B1 protein, indicating that the cyclin destruction machinery has been activated. Nevertheless, the possibility remains that premature chromosome condensation results in damage that blocks mitotic exit through some as yet undefined mechanism. We attempted to rule out this possibility by testing the effects of Cdc2AF and cyclin B1 on mitotic exit in cells that were first arrested in mitosis with a microtubule drug (nocodazole). Despite repeated attempts with a wide range of MOIs, we were unable to obtain significant foreign protein expression in cells arrested in mitosis.

Postmitotic kinase activity in cells expressing wild-type cyclin B1 does not decline as rapidly or as far as it does in control cells (Fig. 1 *B*, *b*), suggesting that mechanisms in addition to inhibitory Cdc2 phosphorylation must contribute to mitotic exit in these cells. Exclusion of cyclin B1 from the nucleus may be such a mechanism. Cells expressing NLS-tagged cyclin B1 consistently displayed a partial defect in the exit from mitosis: ~20% of these cells remained arrested with condensed chromosomes (Fig. 1 *D*). Cells coexpressing nuclear cyclin B1 and Cdc2AF were almost completely arrested in a mitotic state (Fig. 1 *D*) that was always accompanied by nuclear envelope breakdown (data not shown). Cyclin B1-associated kinase activity also remained at high levels in these cells (Fig. 1 *B*, *c* and *f*). Thus, under conditions where cyclin degradation is compromised, exclusion of cyclin B1 from the newly forming nucleus may be required for efficient inactivation of Cdc2 and to prevent rapid reentry into mitosis.

Nuclear Targeting of Cyclin B1 Controls Mitotic Entry After DNA Damage

We next addressed our hypothesis that DNA damage acts in part by maintaining the cytoplasmic localization of cyclin B1. HeLa cells were arrested at the G1/S boundary, infected with various recombinant adenovirus vectors, and released from the arrest. 1 h after release, cells were irradiated with 6 Gray of x rays to induce DNA damage. Under these conditions, control cells exhibited a G2 arrest of variable length, and then began passing asynchronously into mitosis ~16 h after release from G1/S (Fig. 3, *B–D*). Cdc2 was phosphorylated at inhibitory sites during the damage-induced arrest (Fig. 3 *A*). Because the passage through mitosis was asynchronous, Cdc2 dephosphorylation and cyclin B1 degradation in the population occurred more gradually than in unirradiated cells (compare Fig. 3 *A* and Fig. 1 *B*). Cells expressing wild-type cyclin B1 exhibited a slight advancement in the onset of mitosis after DNA damage (Fig. 3, *B–D*). The presence of exogenous cyclin B1 in damaged cells was accompanied by more extensive inhibitory phosphorylation of Cdc2 (Fig. 3 *A*), presumably due to increased quantities of cyclin-bound Cdc2.

Expression of NLS-tagged cyclin B1 caused striking premature mitotic events after DNA damage (Fig. 3, *B–D*). PCC was observed in over 25% of cells 8 h after the G1/S release, and quickly rose to include the majority of cells 12–16 h after release (Fig. 3 *D*). The continued chromosome condensation in these cells, which was accompanied by nuclear envelope breakdown (data not shown), suggests that many of these cells were arrested in a mitotic state. Premature Cdc2 dephosphorylation and cyclin B1 degradation also occurred in these cells (Fig. 3 *A*). Thus, the damage-induced G2 delay was significantly reduced in cells expressing nuclear cyclin B1. These cells also exhibited a pronounced defect in mitotic exit.

NLS-tagged cyclin B1 tended to be expressed in these experiments at slightly (about twofold) higher levels than wild-type cyclin B1. To rule out the possibility that this difference in protein levels was responsible for the difference in the effects of the two proteins, we performed additional experiments in which the expression of wild-type cyclin B1 was doubled by increasing the multiplicity of viral infec-
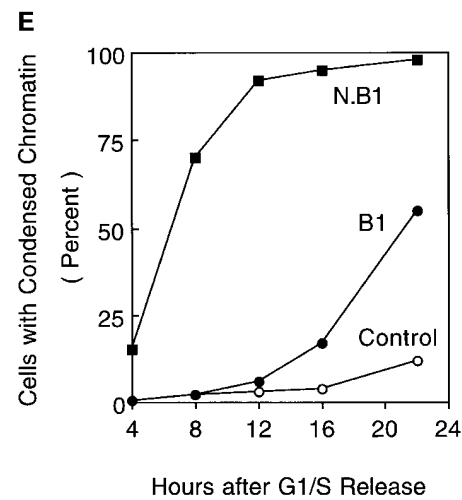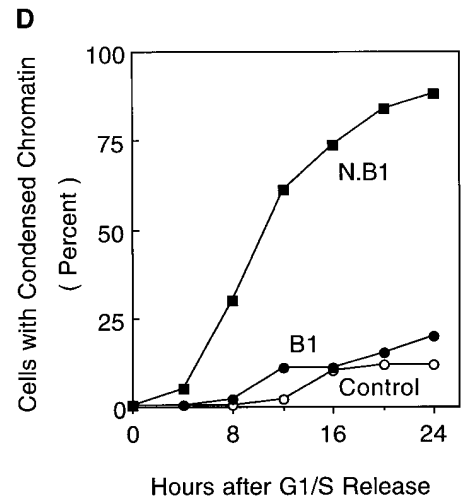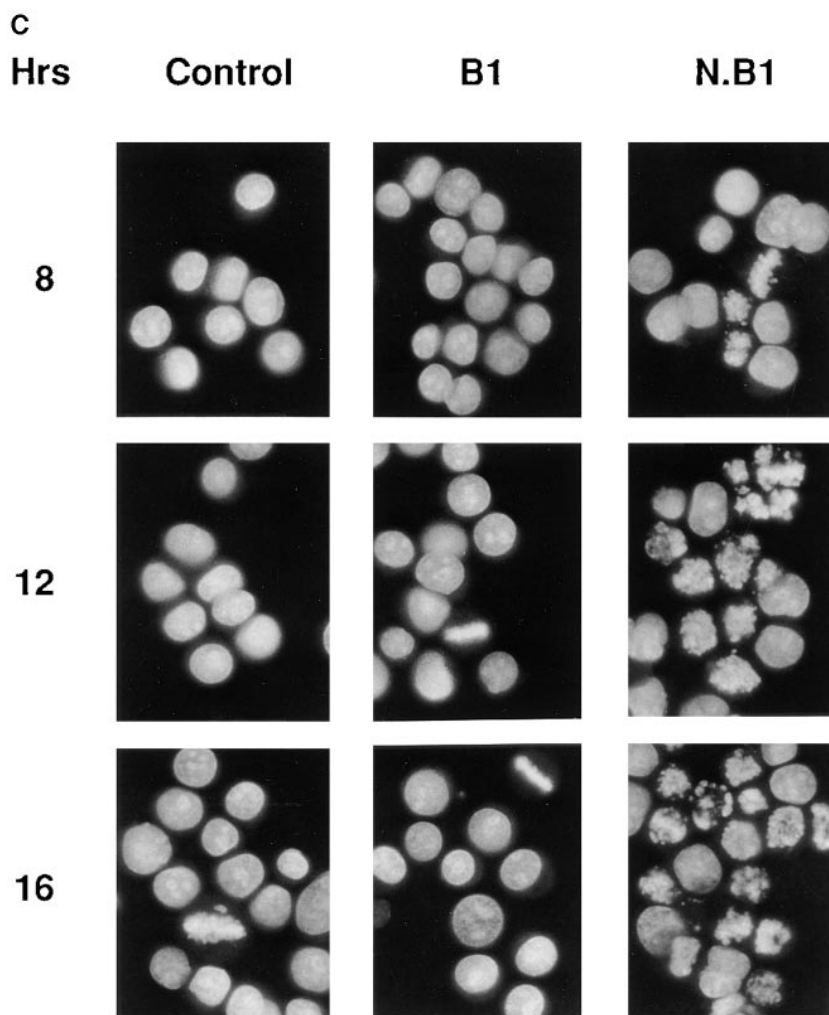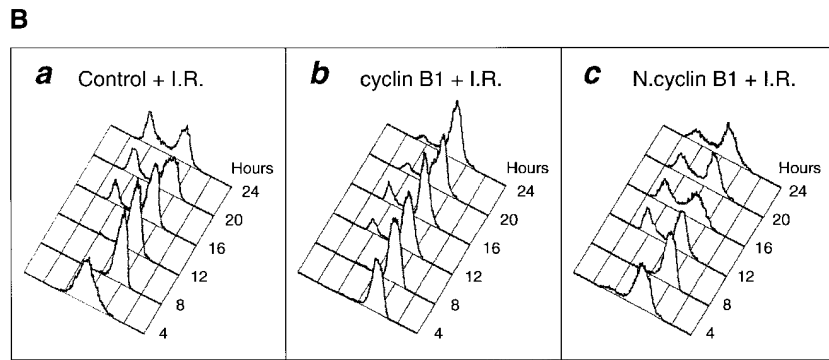
A

B

a Control + I.R.   *b* cyclin B1 + I.R.   *c* N.cyclin B1 + I.R.

C
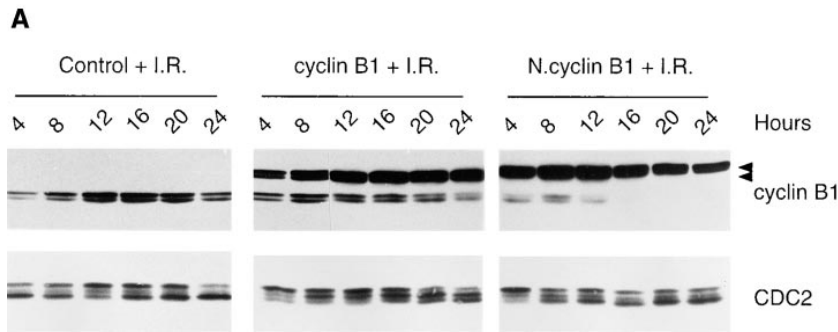
Hrs   Control   B1   N.B1

D

E

Figure 3. Nuclear cyclin B1 reduces the G2 arrest after DNA damage. (*A*) HeLa cells synchronized at the G1/S boundary were infected with adenoviruses encoding tTA (left, control), tTA plus cyclin B1 (*middle*), or tTA plus NLS-cyclin B1 (*right*). 1 h after the G1/S release, cells were x-irradiated with ∼6 Gray. Cell lysates prepared at the indicated times were subjected to immunoblotting with anti-cyclin B1 (*top*) and anti-PSTAIRE antibodies (*bottom*). Arrowheads indicate virally-encoded cyclin B1 and NLS-tagged cyclin B1. I.R., ionizing radiation. (*B*) Half of the cells from the experiment in *A* were subjected to flow cytometric analysis of DNA content. (*C*) Samples of propidium iodide–stained cells from selected times points were analyzed by microscopy and photographed to illustrate the premature chromosome condensation morphology in cells expressing NLS-tagged cyclin B1. (*D*) Percentage of cells with premature chromosome condensation was determined by counting at least 300 cells for each sample. (*E*) A separate experiment was performed in which the microtubule-destabilizing drug nocodazole was added to the cell culture medium after the G1/S release; this results in accumulation of all cells in mitosis. The percentage of cells with premature chromosome condensation was quantitated as in *D*. Note that the radiation dose in this experiment was slightly greater than that in *D*, resulting in a more prolonged G2 arrest in control cells.

tion. This increase did not lead to significant increases in the frequency of premature mitotic events (data not shown).

The timing of mitosis in control and cyclin B1–expressing cells was difficult to evaluate in these experiments because of their asynchronous passage through mitosis, and because they did not accumulate in a mitotic state like the nuclear cyclin B1–expressing cells. We therefore performed an additional experiment with cells released from the G1/S arrest and irradiated in culture medium containing nocodazole (Fig. 3 *E*) to arrest cells in the following mitosis. In this experiment, x irradiation resulted in a more prolonged G2 delay in control cells, and mitotic cells began to accumulate over 16 h after G1/S release. Cells expressing cyclin B1 began to enter mitosis after 12 h. As before, expression of NLS-tagged cyclin B1 resulted in extensive premature mitotic events (Fig. 3 *E*).

Cdc2 Dephosphorylation and Nuclear Cyclin Import are the Key Mechanisms Governing Mitotic Entry After DNA Damage

To assess the relative importance of Cdc2 dephosphorylation and nuclear cyclin import in the timing of mitosis after DNA damage, we compared mitotic timing in damaged cells expressing Cdc2AF and NLS-tagged cyclin B1, alone or in combination.

In the experiment shown in Fig. 4, x-irradiated control cells began to enter mitosis asynchronously after 12 h (Fig. 4 *C*). As before, expression of NLS-tagged cyclin B1 induced a partial override of the damage-induced G2 delay. In this experiment the fraction of cells with PCC rose to 50% by 16 h (Fig. 4, *B* and *C*); PCC was again accompanied by Cdc2 dephosphorylation and cyclin degradation (Fig. 4 *A*). Expression of Cdc2AF also resulted in partial bypass of the damage-induced G2 arrest, as seen in our previous work (Jin et al., 1996). Coexpression of Cdc2AF and NLS-tagged cyclin B1 resulted in extensive premature mitotic events (Fig. 4 *C*). Wild-type cyclin B1 was also very potent in the presence of Cdc2AF, despite its minimal effects when expressed alone. Thus, combinations of Cdc2AF and either version of cyclin B1 induced premature mitotic events to about the same extent as they did in cells lacking DNA damage (Fig. 1), suggesting that these combinations are able to abolish completely the damage-induced G2 delay. Coinfection also leads to an arrest in a mitosis-like state with condensed chromosomes, variable DNA content, and degraded endogenous cyclin.

Discussion

Control of Mitotic Initiation

Our results indicate that the translocation of cyclin B1 from the cytoplasm to the nucleus is a rate-limiting step in the initiation of human mitosis, particularly under conditions where inhibitory phosphorylation of Cdc2 is disabled. Cdc2AF expression or nuclear cyclin B1 targeting each have a minor effect on mitotic entry under normal growth conditions, but together are capable of inducing extensive premature mitotic events (Fig. 1). A simple explanation for these results is that the effects of Cdc2AF are limited by cytoplasmic localization of cyclin B1, while the effects of nuclear cyclin B1 are limited by inhibitory phosphorylation of Cdc2. When both protective mechanisms are bypassed, mitotic catastrophe results.

In previous work, coexpression of Cdc2AF and wild-type cyclin B1 led to premature mitotic events in asynchronous cells (Heald et al., 1993). We also found that wild-type cyclin B1 has moderate effects on the timing of mitosis in the presence of Cdc2AF (Fig. 1), confirming that the effects of Cdc2AF are limited by cyclin B1 concentrations. However, in most experiments we found that the effects of wild-type cyclin B1 were consistently less pronounced than those seen with NLS-tagged cyclin B1. We therefore conclude that premature mitotic events in our experiments are caused not simply by increased concentrations of cyclin B1, but also by premature nuclear localization of the protein.

Control of Mitotic Exit

Recent studies in the budding yeast *Saccharomyces cerevisiae* suggest that inhibitory phosphorylation of Cdc28, the major CDK in this organism, can contribute to mitotic exit under some conditions (Minshull et al., 1996). Our results suggest that similar mechanisms may exist in human cells. We found that cells expressing a stabilized form of cyclin B1 were capable of exiting mitosis, and that increased Cdc2 phosphorylation accompanied mitotic exit (Fig. 1). Coexpression of Cdc2AF with the stabilized cyclin B1 resulted in an apparent defect in the ability of cells to exit mitosis (Figs. 1 and 2). We also observed a minor defect in mitotic exit in cells expressing Cdc2AF alone. We conclude that inhibitory phosphorylation of Cdc2 may be involved in the exit from mitosis under these conditions.

How do we reconcile our results with previous evidence that nondegradable cyclin mutants induce a mitotic arrest? One simple explanation is based on the fact that cyclin degradation was only partially defective in our experiments. Partial cyclin destruction may promote mitotic exit by triggering secondary Cdc2 inactivation mechanisms. For example, a partial decrease in Cdc2 activity could result in some dephosphorylation of Wee1 and Cdc25C, further inactivating Cdc2 by phosphorylation and triggering an autocatalytic feedback loop that enhances Cdc2 phosphorylation. Even in cells where cyclin degradation is normal, this auxiliary inactivation loop would be expected to enhance the rapid switch-like behavior of Cdc2 inactivation.

We speculate that exclusion of Cdc2-cyclin B1 complexes from the newly forming nucleus also contributes to the exit from mitosis in cells expressing stabilized cyclin B1. Prolonged chromosome condensation and nuclear envelope breakdown, suggesting a mitotic arrest, were observed in ~20% of cells expressing NLS-tagged cyclin B1 under normal conditions (Fig. 1 *D*) and in almost all of these cells after DNA damage (Fig. 4 *C*). In cells coexpressing nuclear cyclin B1 and Cdc2AF, mitotic exit was completely blocked. In these cells, partial inactivation of Cdc2 may have initiated reformation of the nuclear envelope, but the presence of active Cdc2-cyclin B1 complexes inside the envelope forced a return to the mitotic state.

In several experiments, we found that cells with prolonged PCC at later time points displayed a 2n DNA content (Fig. 1 *C, e* and *f*; Fig. 3 *B, c*; Fig. 4 *B, d–f*). These cells
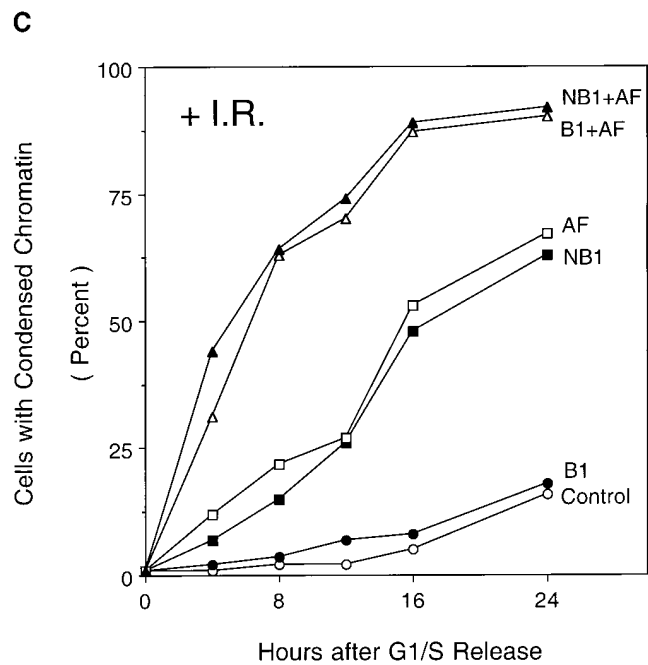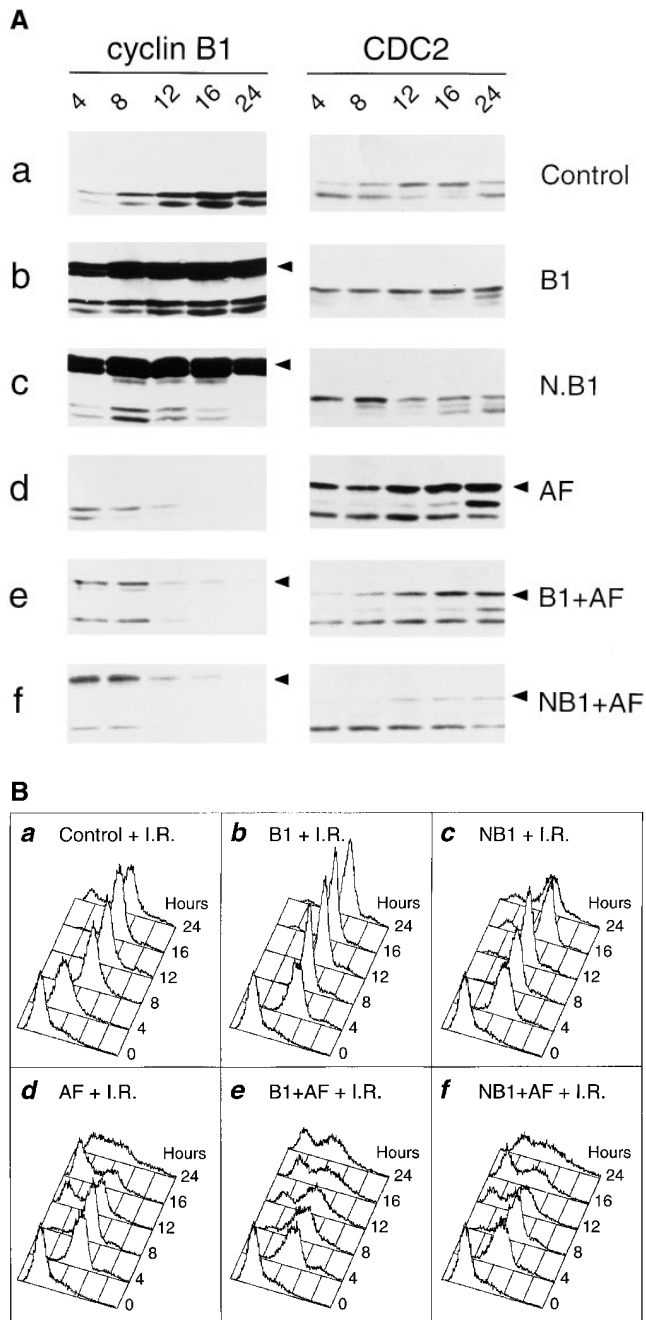
Figure 4. Effects of nuclear cyclin B1 and Cdc2AF on the DNA damage response. (*A*) HeLa cells arrested at G1/S were infected with recombinant adenoviruses encoding tTA (*a*, control) or both tTA and the indicated proteins (*b–f*, abbreviated as in Fig. 1). 1 h after release from the G1/S block, cells were x-irradiated with ∼6 Gray. At the indicated times, cell lysates were prepared and subjected to immunoblotting with anti-cyclin B1 and anti-PSTAIRE antibodies. Arrowheads indicate virally encoded proteins. (*B*) Half of the cells from the experiment in *A* were fixed, stained with propidium iodide, and subjected to flow cytometry to determine DNA content. I.R., ionizing radiation. (*C*) Propidium iodide–stained cells from the analysis in *B* were examined by fluorescence microscopy, and the percentage of cells with premature chromosome condensation was determined by counting at least 300 cells per sample. In a parallel experiment, analysis of Myc-tagged cyclin localization confirmed that cyclin B1 and NLS-cyclin B1 were cytoplasmic and nuclear, respectively, in DNA-damaged interphase cells, either in the presence or absence of Cdc2AF (data not shown, but results were similar to those in Fig. 1 *A*).

apparently achieved exit from mitosis, and then immediately reentered a mitotic state with condensed unreplicated DNA.

Mitotic Entry after DNA Damage

Previous studies clearly suggest that the G2 arrest caused by DNA damage in human cells involves stabilization of the inhibitory phosphorylation sites on Cdc2. Expression of Cdc2AF in HeLa cells reduces the damage-induced G2 delay (Jin et al., 1996; this work), and increases radiation sensitivity (Blasina et al., 1997). The DNA damage response in human cells probably requires the protein kinase Chk1, which has recently been shown to phosphorylate Cdc25C at Ser216, inhibiting its function in vivo (Peng et al., 1997; Sanchez et al., 1997; Weinert, 1997). Inducible expression of a Cdc25C S216A mutant partially reduces the damage-induced cell cycle delay (Peng et al., 1997). Thus, DNA damage appears to enhance Cdc2 phosphorylation by inhibiting the phosphatase Cdc25C.

Expression of Cdc2AF does not completely abolish the G2 arrest that follows DNA damage (Jin et al., 1996; this work); in addition, this arrest is accompanied by high levels of cytoplasmic Cdc2-cyclin B1 activity (Smeets et al., 1994; Jin et al., 1996). Thus, mechanisms in addition to Cdc2 phosphorylation must delay mitosis after damage. The present results raise the possibility that DNA damage acts in part by stabilizing the cytoplasmic localization of cyclin B1. We found that constitutive nuclear targeting of cyclin B1 caused a significant reduction in the damage-

induced G2 delay, and a combination of nuclear cyclin B1 and Cdc2AF in damaged cells resulted in a premature mitotic catastrophe similar to that seen in cells lacking damage.

As in undamaged cells, the total concentration of cyclin B1, as well as its localization, has an impact on the DNA damage response. Under some conditions, DNA damage transiently reduces cyclin B1 expression in HeLa cells (Maity et al., 1995), and damage-induced arrest is reduced in cells overexpressing cyclin B1 (Kao et al., 1997). We also observed a moderate reduction in the damage-induced arrest in cells expressing wild-type cyclin B1, and a dramatic reduction in cells coexpressing cyclin B1 and Cdc2AF (Fig. 4 *C*). Nevertheless, in the absence of Cdc2AF, the effects of NLS-tagged cyclin B1 were clearly more extensive than those of wild-type cyclin B1.

Little is known about the relationship between Cdc2-cyclin B1 activation and nuclear translocation. In starfish eggs, Cdc2 activation precedes nuclear import (Ookata et al., 1992). Similarly, careful analysis of cyclin B1 localization in HeLa cells suggests that import occurs at about the time or just after cytoplasmic changes in microtubule behavior and centrosome separation (Bailly et al., 1992). Thus, it seems likely that nuclear import in human cells either immediately follows Cdc2 activation or is somehow interdependent with it. Therefore, it is possible that nuclear translocation is simply a consequence of Cdc2 activation, and is not a separately regulated process. This possibility is not consistent with our observations that Cdc2AF does not completely bypass the DNA damage arrest, and that the damage-arrested cells contain abundant, cytoplasmic Cdc2-cyclin B1 activity (Smeets et al., 1994; Jin et al., 1996). These observations indicate that Cdc2 activation alone is not sufficient to trigger nuclear import under these conditions, although it remains possible that Cdc2 activation is required for import to occur.

Although our results are consistent with the possibility that DNA damage can separately regulate Cdc2 activation and cyclin localization, proof of this possibility will require a complete understanding of the regulatory pathways controlling localization. These pathways remain mysterious. A region near the amino terminus of cyclin B1 contains a cytoplasmic retention signal (CRS), whose deletion results in constitutive nuclear localization; transfer of this sequence to a nuclear protein results in cytoplasmic localization (Pines and Hunter, 1994). The CRS may act during interphase as a cytoplasmic anchor or a nuclear export signal. In frog eggs, the CRS contains several mitotic phosphorylation sites, and mutation of these sites results in constitutive cytoplasmic localization (Li et al., 1997), suggesting that phosphorylation of the CRS during mitosis inactivates its function, and thereby triggers nuclear translocation. The protein kinases that catalyze cyclin phosphorylation have not been identified. A complete understanding of mitotic entry in normal and damage-delayed cells will require more extensive knowledge of the protein components controlling cyclin B1 localization, their relationship to components governing Cdc2 phosphorylation, and their regulation after DNA damage.

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