Role of Inhibitory CDC2 Phosphorylation in Radiation-induced G2 Arrest in Human Cells

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Abstract. The activity of the mitosis-promoting kinase CDC2-cyclin B is normally suppressed in S phase and G2 by inhibitory phosphorylation at Thr14 and Tyr15. This work explores the possibility that these phosphorylations are responsible for the G2 arrest that occurs in human cells after DNA damage. HeLa cell lines were established in which CDC2AF, a mutant that cannot be phosphorylated at Thr14 and Tyr15, was expressed from a tetracycline-repressible promoter. Expression of CDC2AF did not induce mitotic events in cells arrested at the beginning of S phase with DNA synthesis inhibi-

tors, but induced low levels of premature chromatin condensation in cells progressing through S phase and G2. Expression of CDC2AF greatly reduced the G2 delay that resulted when cells were X-irradiated in S phase. However, a significant G2 delay was still observed and was accompanied by high CDC2-associated kinase activity. Expression of wild-type CDC2, or the related kinase CDK2AF, had no effect on the radiation-induced delay. Thus, inhibitory phosphorylation of CDC2, as well as additional undefined mechanisms, delay mitosis after DNA damage.

IN eukaryotic cells, the initiation of mitosis requires the activity of the cyclin-dependent protein kinase, CDC2 (Nurse, 1990; Dunphy, 1994; King et al., 1994). CDC2 activation is a complex process that begins with the binding of CDC2 to a positive regulatory subunit, cyclin B, whose levels gradually increase during S phase and G2 and reach a maximum at mitosis. Before mitosis, it is thought that the CDC2-cyclin B complex is held in an inactive state by phosphorylation at Thr14 and Tyr15, which is catalyzed by Wee1 and related protein kinases (Nurse, 1990; Dunphy, 1994; King et al., 1994; Mueller et al., 1995). At the end of G2, abrupt dephosphorylation of these sites by the phosphatase CDC25 triggers CDC2 activation.

Dephosphorylation of inhibitory sites in CDC2 is thought to be a key determinant of mitotic timing in the fission yeast, *Schizosaccharomyces pombe*, where genetic defects that reduce Tyr15 phosphorylation lead to premature mitosis (Gould and Nurse, 1989). The timing of mitosis during postblastoderm development in *Drosophila* is also controlled by dephosphorylation of inhibitory sites (Edgar and O'Farrell, 1989, 1990). The importance of inhibitory phosphorylation is less clear in mammalian cells, where premature mitotic events occur to limited extents in transiently transfected cells overexpressing CDC2AF, a mutant form of CDC2 in which Thr14 and Tyr15 are changed to alanine and phenylalanine, respectively (Krek and Nigg, 1991; Heald et al., 1993). In the budding yeast,

Saccharomyces cerevisiae, mutation of the equivalent residues has no clear impact on normal cell cycle timing (Amon et al., 1992; Sorger and Murray, 1992; Booher et al., 1993; Stueland et al., 1993), although these mutations disrupt the coordination of mitosis with bud emergence (Lew and Reed, 1995).

Cell cycle checkpoints normally ensure that mitosis does not occur until DNA replication is complete (Murray, 1994*a,b*). In *S. pombe*, preventing phosphorylation of Tyr15 leads to mitosis even in the presence of chemical inhibitors of DNA synthesis, suggesting that incomplete DNA replication normally restrains mitosis by stabilizing Tyr15 phosphorylation (Enoch and Nurse, 1990). However, in *S. cerevisiae* (Amon et al., 1992; Sorger and Murray, 1992; Booher et al., 1993; Stueland et al., 1993) and in the early embryos of *Xenopus laevis* (Kumagai and Dunphy, 1995), there is evidence that dephosphorylation of the inhibitory sites is not sufficient to induce premature mitosis when DNA synthesis is blocked.

DNA damage also blocks the initiation of mitosis. In both fission and budding yeasts, the cell cycle arrest induced by DNA damage is not affected when inhibitory phosphorylation is prevented, suggesting that some other mechanism delays mitosis under these conditions (Amon et al., 1992; Sorger and Murray, 1992; Barbet and Carr, 1993; Booher et al., 1993; Sheldrick and Carr, 1993; Stueland et al., 1993). In mammalian cells arrested in G2 by DNA damage, some fraction of the CDC2 in the cell is phosphorylated at the inhibitory sites (Hofmann et al., 1994; Kharbanda et al., 1994; Herzinger et al., 1995; Paules et al., 1995), but it is not known if this phosphorylation is required for G2 arrest.

This work addresses the requirement for inhibitory CDC2 phosphorylation in the mitotic delay that occurs after DNA damage in mammalian cells. We have developed human cell lines in which we can inducibly express the CDC2AF mutant. Expression of this protein results in a substantial decrease in the mitotic delay that occurs after DNA damage, indicating that inhibitory phosphorylation does play a role in CDC2 regulation under these conditions. However, expression of CDC2AF does not completely abolish G2 arrest, indicating that additional inhibitory mechanisms also restrain mitosis after DNA damage.

Materials and Methods

Cell Lines

Hemagglutinin (HA)[1] epitope-tagged CDC2, CDC2AF, and CDK2AF were constructed as described (Desai et al., 1992; Gu et al., 1992) and subcloned into pUHD10-3, in which they are driven by a *tet* operator fused to a minimal CMV promoter (Gossen and Bujard, 1992). Plasmids were cotransfected with a hygromycin resistance plasmid into HtTA-1 cells, a HeLa cell derivative containing the pUHD15-1 neo plasmid expressing the *tTA* transactivator under the control of a CMV promoter (Gossen and Bujard, 1992). Clones resistant to both neomycin and hygromycin were screened for inducible expression of HA-tagged CDC2 and CDK2.

Cell Cycle Synchronization and X-Irradiation

Hela cells were synchronized with a double thymidine block protocol. Exponentially proliferating cells maintained in DME/10% FCS/10 μg/ml tetracycline were arrested in S phase by treatment with thymidine (2 mM) for 17 h, and were released from the arrest by three washes 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). Cells were grown in fresh medium for 9 h and then subjected to a second thymidine treatment. After 14 h, cells were washed three times with PBS to allow progress into S phase. In the half of the population where foreign protein expression was desired, tetracycline was removed throughout the entire synchronization, from the beginning of the first thymidine treatment. For ionizing radiation experiments, cells released from the G1/S block for 1 h were X-irradiated at room temperature with a 150-kV Philips X-ray machine without a filter at a rate of 2.5 Gray/min for a total dose of 6 Gray.

For flow cytometric analysis of DNA synthesis and DNA content, cells were cultured in medium containing 10 μM bromodeoxyuridine (BrdU) for 60 min before harvesting. Cells were trypsinized, fixed in 70% ethanol, and stained with FITC-conjugated α-BrdU antibodies and propidium iodide (Larsen, 1994). Stained cells were analyzed by a FACScan (Becton Dickinson, Mountain View, CA), and quantitation of S phase cells (labeled by the FITC-conjugated α-BrdU) and G1 and G2/M cells (assessed by DNA content) was accomplished by a multiparameter assay with Lysis II software.

Chromosome Spreading

Chromosome spreading was performed as described (Heald et al., 1993). Briefly, rounded cells were gently collected by rinsing the dishes once with warm cell culture medium. The cells were pelleted and swollen by incubation in 10 ml of hypotonic buffer (75 mM KCl) for 8 min. Swollen cells were centrifuged and fixed in methanol/acetic acid (3:1, vol/vol) for 8 min. Drops of fixed cells were dropped from a distance of 1 m onto the surface of glass slides. Slides were dried and stained with HOECHST 33258.

Biochemical Procedures

To prepare cell lysates, cells were detached from 10-cm dishes with a rubber policeman, harvested by centrifugation, and resuspended in 200 μl lysis buffer (10 mM Hepes 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$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 20 U/ml aprotinin). Ly-

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1. *Abbreviation used in this paper*: HA, hemagglutinin.

sates were clarified by centrifugation (10,000 g, 10 min, 4°C). To measure histone H1 kinase activity, cell lysate (50 μg) was incubated for 1 h at 4°C with monoclonal anti-HA antibody 12CA5 (3 μg), crude rabbit anti-cyclin B serum (0.5 μl) (Pines and Hunter, 1989), or affinity-purified antibody against the COOH terminus of human CDC2 (0.5 μg) (Rosenblatt et al., 1992) bound to protein A–Sepharose (Sigma Chem. Co., St. Louis, MO). Immunoprecipitates were washed three times with HBS (10 mM Hepes, 150 mM NaCl, pH 7.4) + 0.1% Triton X-100, twice with HBS + 1 mM dithiothreitol (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 using the ProtoBlot Western blot AP System (Promega, Madison, WI).

Results

Inducible Expression of CDC2AF

To study the role of inhibitory CDC2 phosphorylation in mitotic timing and the G2 arrest caused by DNA damage,
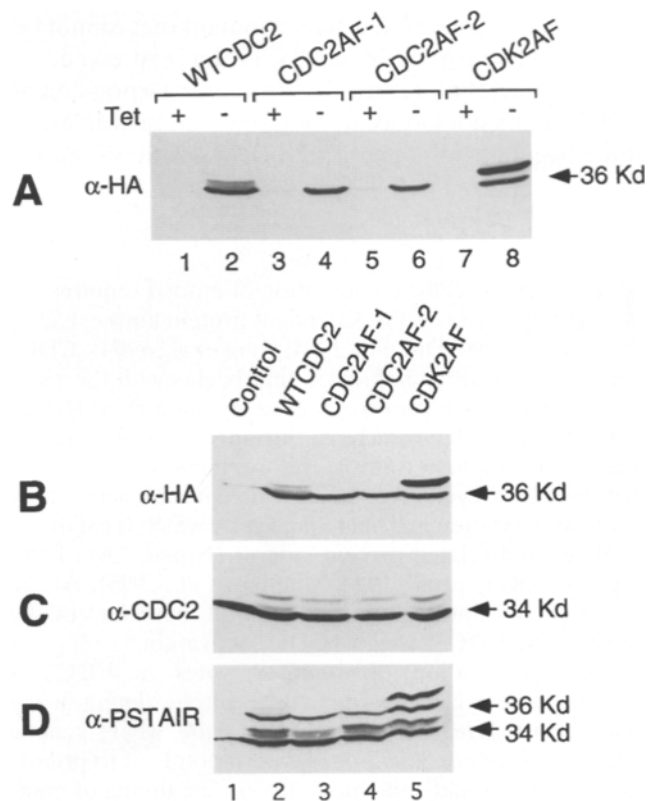

Figure 1. Inducible expression of HA-tagged CDC2 and CDK2 in HeLa cells. (*A*) Cell lines expressing HA-tagged wild-type CDC2, CDC2AF, or CDK2AF were cultured in the presence (*Tet +*) or absence (*Tet −*) of tetracycline (2 μg/ml). Cell lysates (50 μg) were analyzed by immunoblotting with the anti-HA antibody 12CA5. (*B*, *C*, and *D*) Lysates of cells cultured in the absence of tetracycline were immunoblotted with 12CA5 (*B*), anti-CDC2 (*C*), or anti-PSTAIRE (*D*) antibody. Approximate sizes of the bands are indicated on the right. The anti-CDC2 antibody (Rosenblatt et al., 1992) recognizes the COOH terminus of endogenous CDC2 but does not recognize the HA-tagged CDC2. Control lanes contain recombinant CDC2 (50 ng, B–D, lane *1*), expressed and purified from Sf9 cells as described previously (Desai et al., 1992).
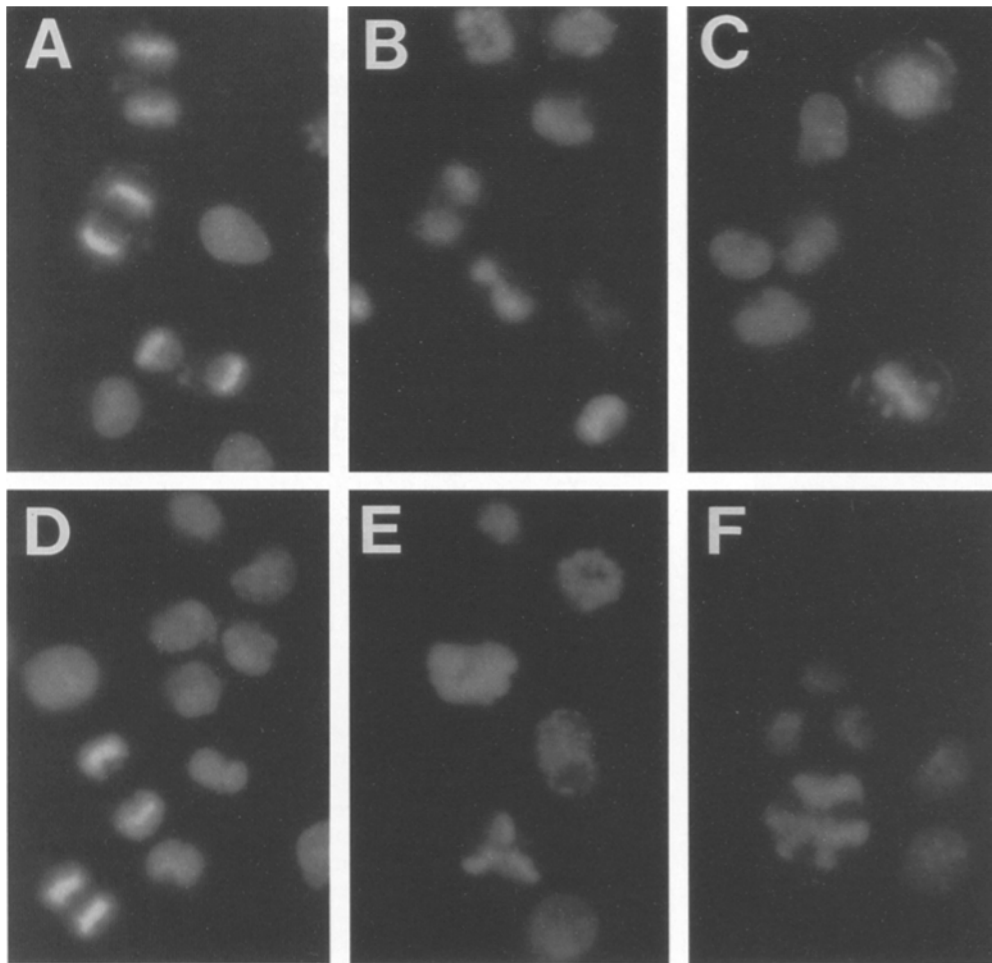
Figure 2. Conditional expression of CDC2AF and CDK2AF induces premature chromatin condensation in some cells. Cells were synchronized at the G1/S boundary by a double thymidine treatment in the presence or absence of tetracycline (2 μg/ml). At various times after release from the G1/S block, cells were detached from tissue culture dishes by trypsinization, fixed in 70% ethanol, stained with HOECHST 33258, and examined by fluorescence microscopy. Photographs represent CDC2AF-1 (*A*, *B*, and *C*) or CDK2AF (*D*, *E*, and *F*) cells 12 h after release from the G1/S block, either in the presence (*A* and *D*) or absence (*B*, *C*, *E*, and *F*) of tetracycline. (*G*) Frequencies of nuclear DNA abnormalities in CDC2AF-1 or CDK2AF clones after release from G1/S block. Cells with aberrant nuclear DNA condensation were scored as a percentage of the total cell nuclei counted. At least 300 cells were counted from each sample.
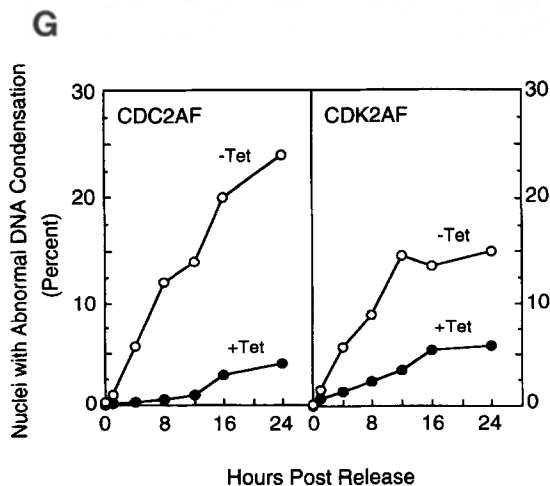
we established stable human HeLa cell lines in which we could conditionally express wild-type human CDC2 or the CDC2AF mutant under the control of a tetracycline-repressible promoter (Gossen and Bujard, 1992). All experiments were performed with two different CDC2AF clones, and identical results were obtained. We also studied cell lines expressing an equivalent AF mutant form of CDK2, a CDK thought to play a role in the initiation or maintenance of DNA replication. All proteins contained a

COOH-terminal epitope tag from influenza hemagglutinin (HA), which is recognized by the 12CA5 monoclonal antibody (Desai et al., 1992). Expression of the various CDKs in these cell lines is very low when cells are cultured in the presence of tetracycline; removal of tetracycline increases expression dramatically (Fig. 1 *A*). Expression is maximal within 12 h of tetracycline removal (not shown). The HA-tagged wild-type CDC2 (Fig. 1 *A*, lane 2), as well as endogenous CDC2 (Fig. 1 *C*), migrated on polyacryl-

D

Percent of Nuclear Spreads

100

50

0

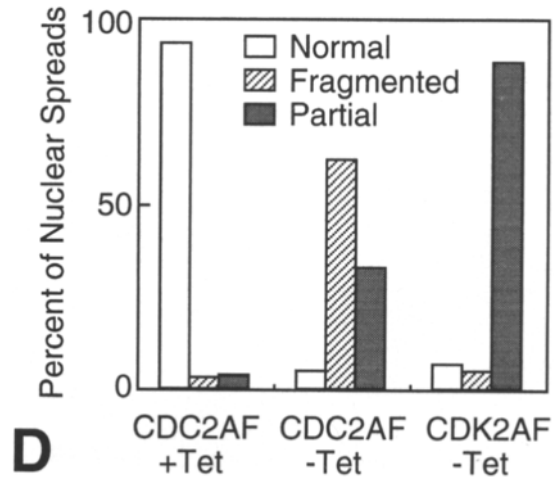□ Normal
▨ Fragmented
■ Partial

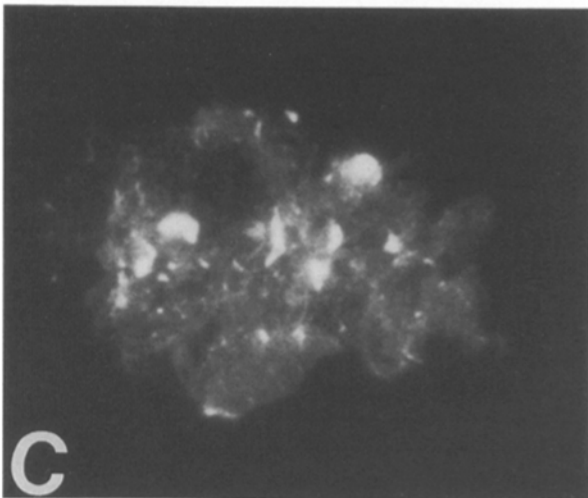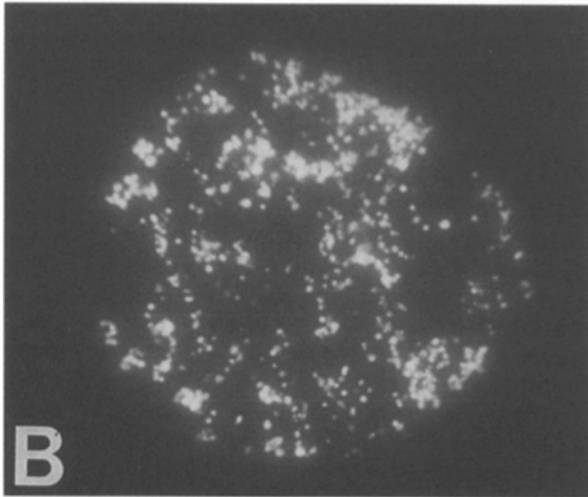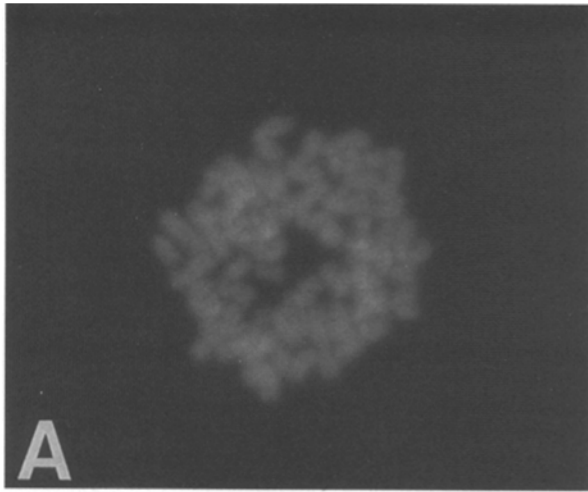CDC2AF
+Tet

CDC2AF
-Tet

CDK2AF
-Tet

Figure 3. Conditional expression of CDC2AF and CDK2AF induces abnormal chromatin spreading. 16 h following release from a G1/S block, rounded cells in the culture were collected by gently rinsing the cell culture dishes with warm medium. This method yields <1% of CDC2AF-1 cells grown in the presence of tetracycline, and ~9% and 6% of CDC2AF-1 and CDK2AF cells, respectively, grown in the absence of tetracycline. Cells were prepared for chromosome spreading as described in Materials and Methods. (*A*) A normal chromosome spread from a CDC2AF-1 cell grown in the presence of tetracycline. (*B*) A chromosome spread showing the extensive fragmentation in CDC2AF-1 cells in the absence of tetracycline. (*C*) Partial chromatin condensation seen in CDK2AF cells in the absence of tetracycline. (*D*) Frequencies of normal and abnormal chromosome spreads as a fraction of total nuclear spreads. Abnormal spreads were scored either as chromosome fragmentation (fragmented, as in *B*) or as partial chromatin condensation (partial, as in *C*).

dogenous CDC2 by immunoblotting cell lysates with antibodies against the conserved PSTAIRE epitope of CDKs (Fig. 1 *D*). CDC2 is the major immunoreactive protein in HeLa cells (the other major PSTAIRE antigen, CDK2, is expressed at about fourfold lower levels). Based on these immunoblots, we estimate that the amount of HA-tagged CDC2 in these lines is approximately half the amount of endogenous CDC2.

CDC2AF Expression Has Minor Effects on Mitotic Timing

We found that conditional expression of either CDC2AF or CDK2AF in an asynchronous population reduced cell survival over a period of several days. Upon removal of tetracycline from the culture medium, a fraction of the cells rounded up and detached, and cell debris gradually accumulated in the culture medium (data not shown). To understand the cell cycle timing of these effects, we performed more detailed analyses of cells synchronized at specific cell cycle stages. Cells were arrested at the beginning of S phase by a double thymidine block in the presence or absence of tetracycline. Flow cytometry revealed that expression of CDC2AF or CDK2AF did not increase the DNA content of arrested cells (see Figs. 4 and 5 below, time zero). Microscopic analysis of cells stained with the DNA specific dye HOECHST 33258 confirmed that expression of the mutant proteins in arrested cells did not re-

amide gels as closely spaced triplets; as demonstrated previously (Norbury et al., 1991; Solomon et al., 1992), upper bands represent Thr14- and Tyr15-phosphorylated protein and are not seen in the HA-tagged CDC2AF mutant (Fig. 1 *A*, lanes *4* and *6*). CDK2AF migrates as a doublet in which the lower band represents protein phosphorylated at the activating residue Thr160 (Gu et al., 1992).

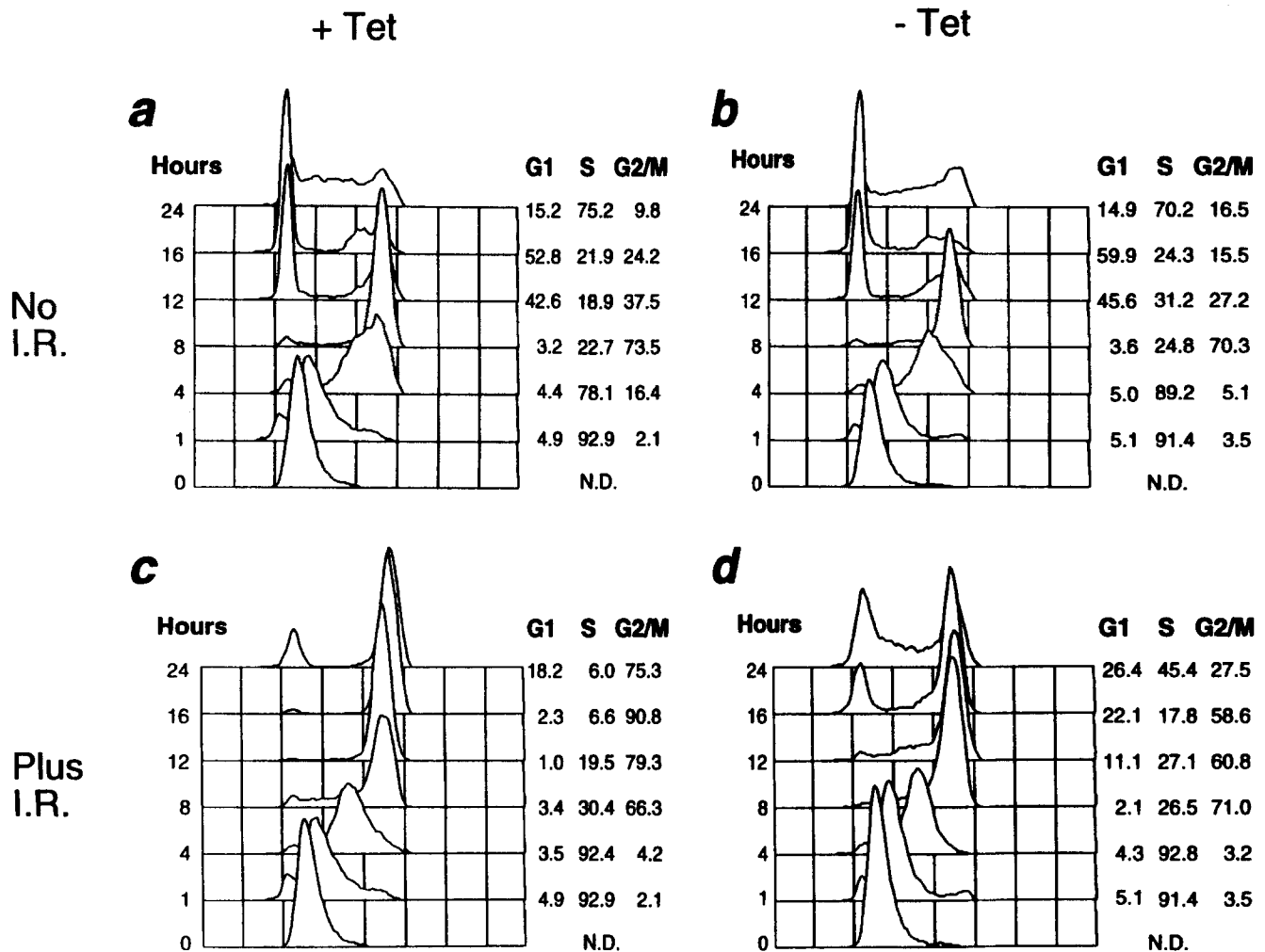We compared the levels of HA-tagged CDC2 and en-

a (No I.R., + Tet)

Hours	G1	S	G2/M
24	15.2	75.2	9.8
16	52.8	21.9	24.2
12	42.6	18.9	37.5
8	3.2	22.7	73.5
4	4.4	78.1	16.4
1	4.9	92.9	2.1
0			N.D.

b (No I.R., − Tet)

Hours	G1	S	G2/M
24	14.9	70.2	16.5
16	59.9	24.3	15.5
12	45.6	31.2	27.2
8	3.6	24.8	70.3
4	5.0	89.2	5.1
1	5.1	91.4	3.5
0			N.D.

c (Plus I.R., + Tet)

Hours	G1	S	G2/M
24	18.2	6.0	75.3
16	2.3	6.6	90.8
12	1.0	19.5	79.3
8	3.4	30.4	66.3
4	3.5	92.4	4.2
1	4.9	92.9	2.1
0			N.D.

d (Plus I.R., − Tet)

Hours	G1	S	G2/M
24	26.4	45.4	27.5
16	22.1	17.8	58.6
12	11.1	27.1	60.8
8	2.1	26.5	71.0
4	4.3	92.8	3.2
1	5.1	91.4	3.5
0			N.D.

Figure 4. Cell cycle progression in CDC2AF-1 cells. Cells were synchronized by double thymidine treatment in the presence (+ Tet, *a* and *c*) or absence (− Tet, *b* and *d*) of tetracycline as in Fig. 2. 1 h after release from G1/S arrest, cells were X-irradiated (6 Gray, Plus I.R.) or received mock irradiation (No I.R.). At the indicated times after G1/S release, cells were incubated with BrdU, harvested, fixed, and incubated with FITC-conjugated anti-BrdU antibodies and propidium iodide as described in Materials and Methods. Stained cells were analyzed by flow cytometry to quantitate S phase cells (labeled by anti-BrdU) and G1 and G2/M cells (assessed by DNA content). DNA content profiles are shown in the figure, and quantitation of G1, S, and G2 cells is shown on the right.

sult in cell rounding or premature chromatin condensation (see Fig. 2 *G* below, time zero).

When cells were released from the G1/S arrest in the absence of tetracycline, abnormally condensed nuclear DNA was observed in a fraction of the population, suggesting that premature chromatin condensation was occurring as the cells progressed through S phase and G2 (Fig. 2). The effects of CDC2AF and CDK2AF were consistently different: CDC2AF induced a slightly higher number of nuclei with hypercondensed chromatin (Fig. 2 *G*), despite being expressed at lower levels than CDK2AF (Fig. 1 *A*). In both cell lines, however, the majority of cells displayed a nuclear DNA-staining pattern indistinguishable from that of the same cells grown in the presence of tetracycline.

To further characterize the nature of the chromosome abnormalities seen in these cell lines, we performed chromosome spreading experiments. Rounded cells were collected 16 h after release from the G1/S block. A normal chromosome spread with intact chromosomes is shown in

Fig. 3 *A*. Spreads prepared from cells expressing CDC2AF displayed extensive chromosome fragmentation (Fig. 3 *B*), which is thought to result from premature mitotic events occurring during S phase (Heald et al., 1993). A qualitatively different pattern of partial chromatin condensation was observed in most of the abnormal spreads prepared from CDK2AF-expressing cells (Fig. 3, *C* and *D*).

Although expression of CDC2AF and CDK2AF induced hypercondensed nuclei in some cells, flow cytometric analysis of DNA content and DNA synthesis in the total cell population revealed that expression of these proteins did not have significant effects on the timing of progression through S phase or mitosis (Fig. 4, *a* and *b*; and Fig. 5, *a* and *b*).

CDC2AF Expression Partially Reduces the G2 Delay Caused by DNA Damage

To test the role of inhibitory CDC2 phosphorylation in

DNA damage-induced mitotic checkpoint control, we X-irradiated cells (6 Gray) during early S phase, 1 h after release from the G1/S arrest. Control cells received mock irradiation. DNA synthesis and DNA content were then measured by flow cytometry (Fig. 4). Unirradiated cells progressed from S to G2 between 4 and 8 h after release from the G1/S block, and completed mitosis between 8 and 12 h after release (Fig. 4, a and b). Irradiation resulted in slower progress through S phase, and induced an ~12 h mitotic delay (Fig. 4 c). Only 18.2% of irradiated cells emerged in G1 24 h after release (Fig. 4 c), while 42.6% of unirradiated cells appeared in G1 as early as 12 h after release (Fig. 4 a).

Expression of CDC2AF resulted in partial abrogation of the radiation-induced G2 delay. In the absence of tetracycline, irradiated cells began to appear in G1 12 h after release (Fig. 4 d), and we estimate that the mitotic delay due to irradiation was reduced from about 12 h in the absence of CDC2AF to ~4–6 h in its presence. Similar results were obtained in three independent experiments with two CDC2AF clones. Only CDC2AF was able to induce this partial bypass of the radiation-induced G2 delay: we failed to detect any change in the delay in cells expressing CDK2AF (Fig. 5, c and d) or wild-type CDC2 (data not shown).

Kinase Activity Associated with CDC2 and Cyclin B during G2 Arrest

Analyses of CDC2 and cyclin B levels and activity were performed to clarify the biochemical changes accompany-

ing cell cycle progression in the above experiments (Fig. 6). In unirradiated cells grown in the presence of tetracycline (Fig. 6, lanes 1–5), CDC2- and cyclin B–associated kinase activities peaked 8 h after G1/S release and declined as cells progressed into G1 (see Fig. 4 a). Expression of CDC2AF resulted in abundant CDC2AF-associated kinase activity (Fig. 6 D, lanes 6–10), which peaked 8 h after G1/S release, and then remained partially elevated after mitosis, perhaps due to an association with cyclin A or B in the subsequent cell cycle. The presence of CDC2AF did not affect the timing of CDC2- and cyclin B–associated activities, which still peaked ~8 h after G1/S release and then declined (Fig. 6, E and F; lanes 6–10). These results are consistent with our flow cytometric evidence that the timing of mitosis in most cells is not affected by CDC2AF expression.

To confirm that CDC2AF activity represents a significant fraction of total CDC2-associated kinase activity, we immunodepleted CDC2AF-HA from lysates of cells harvested 8 h after G1/S release. Incubation with a large excess of anti-HA antibody, followed by adsorption to protein A–Sepharose and centrifugation, resulted in the removal of over 90% of the CDC2AF-HA from the lysate (data not shown). Cyclin B–associated kinase activity in the immunodepleted lysate was ~40% lower than activity in a lysate incubated with beads alone, demonstrating that CDC2AF is responsible for about half of the cyclin B–associated activity in mitotic cells.

Biochemical analyses of irradiated cells provided additional clues about the importance of inhibitory CDC2
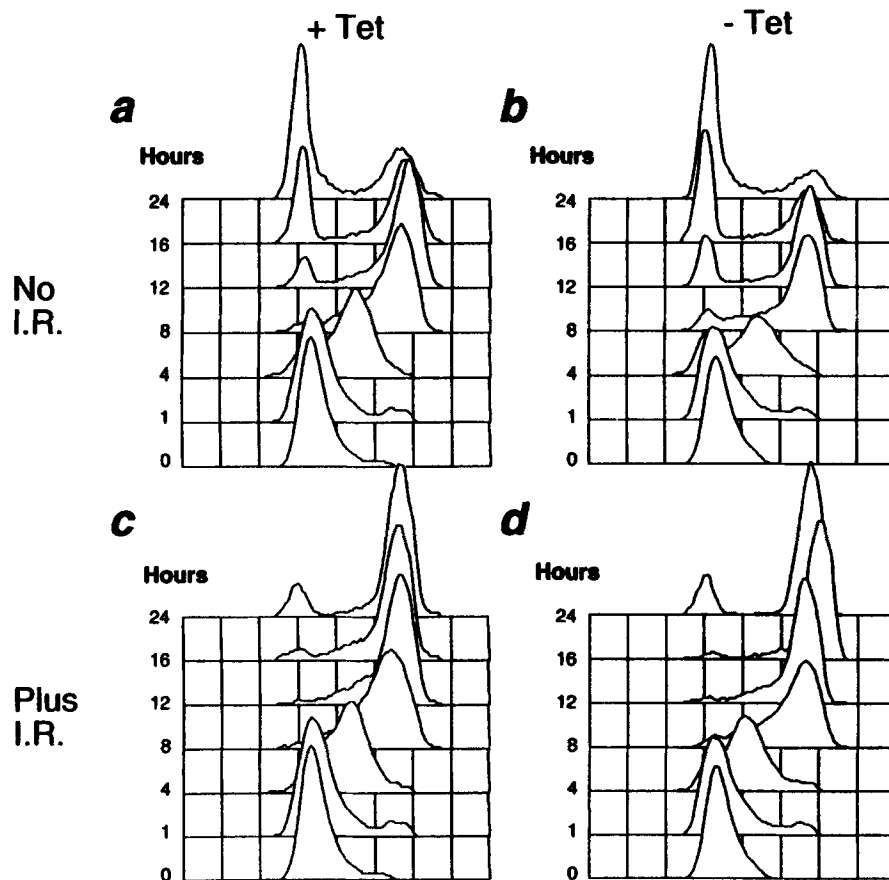

Figure 5. Cell cycle progression in CDK2AF cells. Cells were subjected to the same treatments as those described for CDC2AF-1 in Fig. 4. DNA content profiles are shown for cells at the indicated times after G1/S release, in the presence (+ Tet, a and c) or absence (– Tet, b and d) of tetracycline. Cells received X-irradiation (Plus I.R.) or mock treatment (No I.R.).
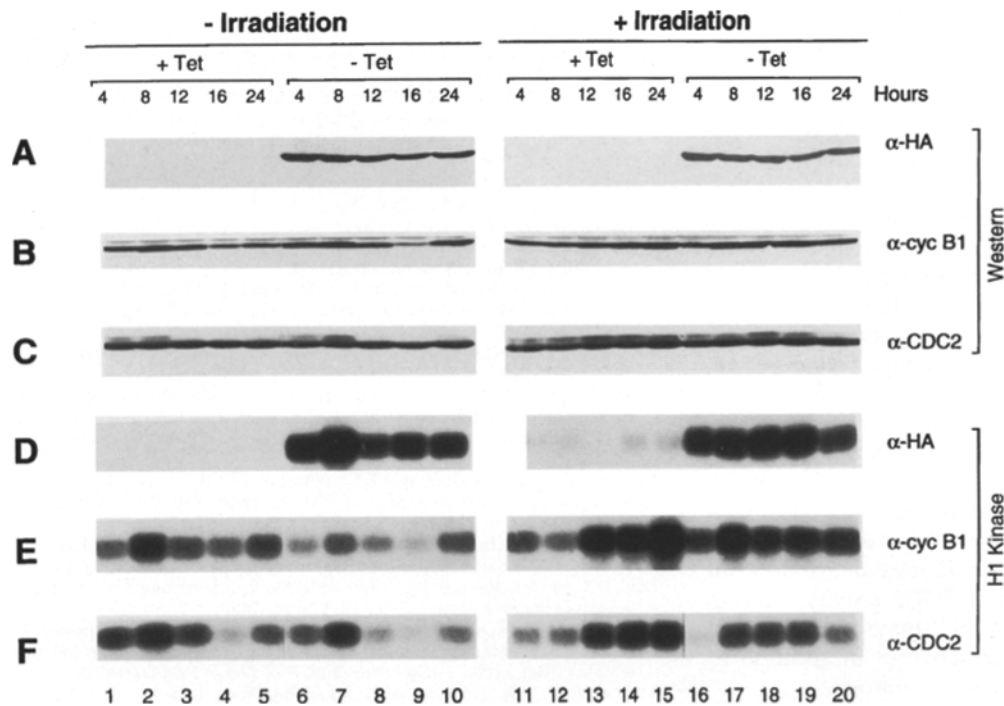
Figure 6. Effect of X-irradiation on levels and activity of CDC2 and cyclin B1. CDC2AF-1 cells were synchronized and irradiated in the presence or absence of tetracycline as described in Fig. 4. At the indicated times after irradiation, cells were lysed and subjected to analysis by immunoblotting with anti-HA (*A*), anti-cyclin B (*B*), and anti-CDC2 (*C*). In addition, histone H1 kinase activity was measured in immunoprecipitates with anti-HA (*D*), anti-cyclin B (*E*), or anti-CDC2 (*F*). Autoradiographic exposure times were 3 h (*D* and *F*) or 1 h (*E*).

phosphorylation in DNA-damage–induced arrest. Radiation-induced G2 arrest was accompanied by reduced gel mobility of a small fraction of the endogenous CDC2 (Fig. 6 *C*, lanes *11–15*), reflecting phosphorylation at Thr14 and Tyr15 as reported previously (Hofmann et al., 1994; Kharbanda et al., 1994; Paules et al., 1995). Irradiation caused a transient delay in the accumulation of cyclin B1 and cyclin B1-CDC2–associated kinase activity (Fig. 6, *B*, *E*, and *F*, compare lanes *2* and *12*), as previously reported (Muschel et al., 1991, 1993; Maity et al., 1995). After this initial delay, however, cyclin B1 levels and cyclin B1-CDC2–associated kinase activity rose to and remained at high levels during the several hours that these cells were delayed in G2 (Fig. 6, *B*, *E*, and *F*; lanes *12–14*; see Fig. 4 *c*). Expression of CDC2AF led to the appearance of large amounts of CDC2AF-associated kinase activity at all time points (Fig. 6, *A* and *D*; lanes *16–20*). Once again, the reduced G2 delay that occurred in the presence of CDC2AF was accompanied by significant amounts of cyclin B1– and CDC2-associated kinase activity (Fig. 6, *E* and *F*, lanes *17* and *18*; see Fig. 4 *c*). Thus, both in the absence and presence of the CDC2AF mutant, DNA-damage–induced G2 arrest was accompanied by high levels of CDC2-associated kinase activity.

Discussion

Our results suggest that inhibitory phosphorylation of CDC2 plays a limited role in the suppression of mitosis in human cells progressing through S phase. Expression of the CDC2AF mutant (or CDK2AF) at approximately normal concentrations did not induce premature mitotic events in cells arrested at G1/S by inhibitors of DNA synthesis, and induced only a low frequency of mitotic events in cells progressing through S phase. Thus, we conclude that phosphorylation of Thr14 and Tyr15 is not solely re-

sponsible for the repression of mitosis when DNA replication is incomplete.

These results are consistent with the results of Heald et al. (1993), who found that transient overexpression of CDC2AF resulted in abnormal chromatin condensation in only 12% of baby hamster kidney cells. We did not observe the high frequency of mitotic events seen in earlier studies of transiently transfected HeLa cells overexpressing CDC2AF (Krek and Nigg, 1991). These discrepancies may be due to differences in the level of CDC2AF expression.

Why are premature mitotic events infrequent in cells containing abundant quantities of active CDC2AF? One potential explanation comes from evidence that the initiation of mitosis is accompanied not just by CDC2 dephosphorylation but by relocalization of cyclin from the cytoplasm to the nucleus (Pines and Hunter, 1991; Ookata et al., 1992; Pines and Hunter, 1994). During S phase and G2, cyclin B (with CDC2 attached) accumulates in the cytoplasm. Just before mitosis, these Cyclin B-CDC2 complexes enter the nucleus and initiate their mitotic functions. The importance of this change of subcellular localization is supported by the studies of Heald et al. (1993), who were able to engineer cells containing abundant quantities of dephosphorylated, active CDC2 whose activity was restricted to the cytoplasm. These active complexes did not induce premature mitotic events. Thus, active CDC2AF-cyclin B complexes may be ineffective in our experiments because they are restricted to the cytoplasm. Indeed, immunofluorescence studies indicate that cells expressing CDC2AF exhibit the normal pattern of cyclin B localization (Jin, P., unpublished results).

Our studies of the G2 arrest after DNA damage provide clear evidence for the importance of inhibitory phosphorylation at this checkpoint. Expression of CDC2AF, but not CDK2AF, resulted in a considerable reduction in the length of the G2 arrest induced by DNA damage. Thus,

we conclude that inhibitory phosphorylation of CDC2 is at least partly responsible for the mitotic delay that occurs after DNA damage.

We have also presented evidence that mechanisms not involving inhibitory phosphorylation of CDC2 contribute to the control of mitosis after DNA damage. First, we observed that although CDC2AF greatly reduced the G2 arrest after DNA damage, there was still a significant mitotic delay of several hours. Second, we found that cells arrested in G2 after DNA damage contain high levels of cyclin B1– and CDC2-associated kinase activity. This observation is reminiscent of the high Cdc28-associated kinase activity in *S. cerevisiae* after DNA damage (Amon et al., 1992; Sorger and Murray, 1992), and raises once again the question of why mitosis is not occurring despite the apparent presence of abundant CDC2 activity. Several possible explanations exist. For example, CDC2 activity may be suppressed in vivo by an inhibitory protein that binds with such low affinity that it is lost during the washing of CDC2 immunoprecipitates. Alternatively, DNA damage may not act through the direct inhibition of CDC2 activity; instead, some function downstream of CDC2 may be blocked. It also remains possible that other activities in addition to CDC2 are required for the induction of mitosis.

The cytoplasmic localization of cyclin B during S phase and G2 might also provide an explanation for the inability of CDC2AF to completely bypass the DNA damage checkpoint. Immunofluorescence studies have shown that cyclin B is primarily cytoplasmic in damage-arrested cells (Smeets et al., 1994)(Jin, P., unpublished results). Thus, DNA damage could act both by stabilizing inhibitory phosphorylation of CDC2 and by maintaining the cytoplasmic localization of cyclin B.

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