CK2 Inhibits Apoptosis and Changes Its Cellular Localization Following Ionizing Radiation

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Abstract
In this study, we show that CK2 (casein kinase II, CKII) participates in apoptotic responses following ionizing radiation (IR). Using HeLa human cervical carcinoma cells, we find that transfection of small interfering RNA against the CK2 α and/or α’ catalytic subunits results in enhanced apoptosis following IR damage as measured by flow cytometry techniques, compared with a control small interfering RNA. Within 2 to 6 hours of IR, CK2 α partially localizes to perinuclear structures, whereas a marked nuclear localization of α’ occurs. Treatment with a pan-caspase inhibitor or transfection of ARC (apoptosis repressor with caspase recruitment domain) suppresses the apoptotic response to IR in the CK2-reduced cells, indicating involvement of caspases. Additionally, we find that CK2 α and/or α’ reduction affects cell cycle progression independent of IR damage in this human cell line. However, the G2-M checkpoint following IR is not affected in CK2 α- and/or α’-reduced cells. Thus, our data suggest that CK2 participates in inhibition of apoptosis and negatively regulates caspase activity following IR damage. (Cancer Res 2005; 65(10): 4362-7)

Introduction
CK2 (formally, casein kinase II) is a protein Ser/Thr kinase complex that forms a heterotetramer mainly consisting of α2β2, αα’β2, or α’2β2, where α and α’ are the catalytic subunits and β is the noncatalytic subunit (for reviews, see refs. 1, 2). The highly conserved amino acid sequences of CK2 from yeast to humans suggest the importance of CK2 in cellular functions, although its major function(s) are not clearly understood. The α and α’ subunits have significant homology in NH2-terminal kinase regions but have diverse COOH-terminal tail regions, which are highly conserved between species. CK2 is essential for basic cell viability because a double knockout of the two catalytic subunits is lethal in yeast (3). However, a single knockout of either α or α’ remains viable, indicating complementary functions of the catalytic subunits, at least with respect to viability in yeast. In male mice, a knockout of CK2 α’ is reported to be sterile and defective in spermatogenesis (4), whereas a knockout of CK2 β shows embryonic lethality in mice (5). CK2 also plays an antiapoptotic role through different pathways, including protection of Bid from cleavage by caspase 8 (6–9). Recently, antitumor activity was found using a peptide that inhibits CK2 activity (10) and through cleavage by caspase 8 (6–9). Recently, antitumor activity was found using a peptide that inhibits CK2 activity (10) and through cleavage by caspase 8 (6–9).

CK2 phosphorylates many substrates that regulate the cell cycle, including p53 (12), Cdc2 (13), and BRCA1 (14). Following UV light damage, CK2 phosphorylates p53 and IκB (15, 16). A role for CK2 has not been determined following ionizing radiation (IR) damage, which principally causes DNA double-strand breaks. Therefore, in this study, we assess the role of CK2 in response to IR damage using human HeLa cervical carcinoma cells. Because a double knockout of α and α’ subunits is lethal and because the functions of α can compensate the functions of α’, we used three different small interfering RNAs (siRNA) against the α and/or α’ subunits. We found that a reduction of CK2 catalytic subunits by siRNA significantly enhanced apoptosis and reduced the G2-M population following IR. However, a G2-M checkpoint arrest following IR damage was not affected by CK2 reduction. We also found that the CK2 α’ subunit translocates into the nucleus within 6 hours of IR treatment, supporting a role for CK2 in response to IR damage.

Materials and Methods
Cell culture, reagents, and transfection of small interfering RNA. HeLa cells were grown in RPMI 1640 supplemented with 10% FCS in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The caspase inhibitor z-VAD was obtained from Biomol (Plymouth Meeting, PA).

The three CK2 siRNAs used were 5’-GAUGACUGACAGCGGUGUdTdT (α siRNA), 5’-UCUAGUGACUGACAGCGGUGUdTdT (α’ siRNA, against α with 100% homology and against α’ with 90% homology), and 5’-CAUGACAGAGGACAGCdTdT (negative control siRNA). Additionally, a Chk1 siRNA (5’-GCGTCAGTCGTAGCTGCGUdTdT) was used as a positive control (17) to assess apoptosis and cell cycle changes following IR. All siRNAs were obtained from Dharmacon (Lafayette, CO). All were annealed with the complementary strand with dTdT overhangs. Unlabeled, the α siRNA (not α 100 siRNA) was used for reduction of the α subunit. Transfection was done with Oligofectamine as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Transfection was done for 30 hours, and HeLa cells were then washed and trypsinized. Next, the cells from one well of a 24-well plate were divided into two to three wells, and RPMI 1640 was added to the culture before irradiation. The efficiencies of transfection were scored using a specific antibody (9E10, Babco, Berkeley, CA). After extraction of protein, total cell extracts were prepared by trichloroacetic acid precipitation to detect CK2 α and α’ using antibodies (N-18 and C-20, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) and to detect Myc using a specific antibody (9E10, Babco, Berkeley, CA). After extraction of trichloroacetic acid with ether, the DNA was sheared by sonication before loading onto gels. An actin antibody (Sigma, St. Louis, MO) was also used for Western blotting as a control.

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Flow cytometry. HeLa cells were fixed with 90% ethanol at −20°C for 60 minutes to a few days, incubated with RNase and stained with propidium iodide, and then subjected to flow cytometry (Coulter, Epics XLMCL, Miami, FL). To measure the G2-M checkpoint response, cells were fixed using 0.25% formaldehyde in the medium before the ethanol treatment and then double-stained with mitotic protein monoclonal-2 antibody (Upstate, Waltham, MA) and propidium iodide. Nocodazole (0.3 mg/ml) was added to the medium and cells were cultured for 24 hours as a general method (18). To perform terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining, cells were also fixed with 0.25% formaldehyde in the medium before the ethanol treatment and then stained with the ApoDirect kit (eBioscience, San Diego, CA).

Confocal microscopy. HeLa cells were irradiated and cultured as described above. At various time points following IR, cells were then fixed with methanol and acetone (1:1) and stained with anti-α or anti-α’ antibodies (N-18 and C-20, respectively; Santa Cruz Biotechnology). Samples were washed, stained with secondary antibodies (Alexa Fluor 633 anti-goat IgG, A21082, Molecular Probes, Eugene, OR) and 4,6-diamidino-2-phenylindole (DAPI), and then subjected to confocal microscopy. Mitotracker (M7514, Molecular Probes) was used for mitochondria staining (30 min, 1 hour). Transfection of endoplasmic reticulum–targeted green fluorescent protein (GFP; ref. 19) was used for staining of the endoplasmic reticulum. The confocal images were obtained with a Zeiss LSM510 inverted confocal microscope system (Carl Zeiss, Oberkochen, Germany) equipped with a tunable T-sapphire laser (Mira-F-V5-XW-220) with a diode pump (Verdi 5 W) and a diode pumped solid state laser and a tunable T-sapphire laser (Mira-F-V5-XW-220) with a diode pump (Verdi 5 W) and a diode pumped solid state laser. All analysis was carried out with Adobe Photoshop Software (Adobe Systems, San Jose, CA).

Gene isolation and site-specific mutagenesis. Genes encoding ARC (apoptosis repressor with caspase recruit domain) and Cdc2 were amplified by a PCR using human placenta total cDNA, and cloned into a pcDNA3-based vector (Invitrogen Life Technologies). The mutations were introduced using Quikchange (Stratagene, La Jolla, CA). All amplified and mutated sequences were confirmed by DNA sequencing in all coding regions.

Results

A sub-G1 population is enhanced in CK2-reduced cell following ionizing radiation. We used a RNA interference technique (20) for reduction of the protein levels of the CK2 catalytic subunits, using transfection of specific siRNAs against the two catalytic subunits α and/or α’. The two antibodies against α or α’ specifically recognized each subunit by Western blotting (Fig. 1A). After siRNA transfection, the protein levels of the CK2 α and α’ subunits were markedly reduced, whereas a control siRNA transfection did not result in changes in protein levels of the CK2 subunits nor actin (control; Fig. 1B). The α10 siRNA was designed to target both α and α’ with 100% and 90% homologies, respectively, and seems to reduce α to a greater extent than α’ (Fig. 1B). The strong inhibition by siRNA continued for at least 48 hours, following siRNA transfection (30 hours) and then 20 hours in complete media (Fig. 1C). The effects of IR treatment in CK2-reduced HeLa cells were assessed for up to 48 hours, based on this continued inhibition of CK2 catalytic unit protein expression (Fig. 1C).

HeLa cells were transfected with siRNA against α and α’ for 30 hours, cultured for 20 hours, and then irradiated to 0, 10, or 30 Gy (time 0). CK2 reduction using siRNA against both α and α’ seemed to result in an increased sub-G1 population at 24 and 48 hours following IR compared with control-transfected cells (Fig. 1D). The control siRNA-transfected HeLa cells showed an IR dose- and time-dependent enhancement in the G2-M population, consistent with a recent report using HeLa cells (21). A single siRNA transfection against either CK2 α or α’ also resulted in an increased sub-G1 population following IR (Fig. 1D), suggesting that each CK2 catalytic subunit is important for inhibition of apoptosis following IR. Additionally, CK2-reduced HeLa cells using both α + α’ siRNA or α’ siRNA alone showed a significant G1 cell cycle delay at 24 hours after 30 Gy IR. These results on cell cycle progression in CK2-reduced HeLa cells are further analyzed (see Fig. 4).

Enhanced apoptosis following ionizing radiation is confirmed by a TUNEL assay in CK2-reduced cells. To further confirm and characterize the apoptotic response following...
IR treatment in the CK2-reduced HeLa cells, we used a TUNEL and propidium iodide double-staining technique with formaldehyde fixation to reduce the sub-G1 population. We found that CK2 reduction in HeLa cells results in a significant TUNEL-positive population with the use of a double (α + α') siRNA transfection even without IR treatment (Fig. 2, top). However, following IR treatment (10 Gy), we also find a time-dependent increase in the apoptotic population in the CK2-reduced cells (24 and 48 hours following IR). In contrast, a very low TUNEL-positive population is found in the (negative) control siRNA-transfected cells with or without treatment with IR. It should be noted that the TUNEL-positive populations do not directly correspond to the sub-G1 populations in Fig. 1D because the TUNEL labeling is an enzymatic treatment to label each cell, whereas a sub-G1 population represents total fragmented DNA detected by flow cytometry.

Use of a single siRNA (α, α', or α10) transfection also shows an increased TUNEL-positive fraction at 48 hours following 10 Gy (Fig. 2, bottom), indicating that the α and α' subunits are independently important for the inhibition of apoptosis following IR damage. We also used a Chk1 siRNA transfection into HeLa cells as a positive control because Chk1 is an essential protein for cell survival and DNA damage response (22, 23). An increased TUNEL-positive fraction is also found in Chk1-reduced HeLa cells (15%), but this fraction is further increased to 55% at 48 hours following 10 Gy (Fig. 2, bottom), similar to the data in CK2-reduced cells.

**CK2 α and α' show differential patterns of cellular localization following ionizing radiation.** To examine the localization of the CK2 α or α' catalytic subunits, we stained nontransfected HeLa cells with anti-CK2 antibodies and analyzed their localization by confocal microscopy. Both CK2 α and α' localized mainly to the cytoplasm in the absence of IR treatment (0 Gy, 24 hours; Fig. 3A). Following IR treatment to 10 Gy and a 24-hour period in complete medium, CK2 α became partially localized to perinuclear regions. The staining patterns of mitochondria (using Mitotracker) did not exclusively correspond to the CK2 α pattern (CK2 α at 10 Gy, a merged panel with yellow color when colocalized; Fig. 3A). Using a higher IR dose (30 Gy) and a longer observation time (48 hours following IR), nuclear fragmentation was seen and CK2 α remained partially localized to perinuclear structures. Further confocal microscopy studies using HeLa cells transfected with endoplasmic reticulum-targeted GFP show that CK2 α is partially localized to the endoplasmic reticulum (data not shown). On the other hand, CK2 α' markedly changed its localization to the nucleus following IR treatment at 10 Gy and culture for 24 hours, whereas a higher dose (30 Gy) and longer observation (48 hours following IR) showed persistent nuclear localization with evidence of nuclear fragmentation (Fig. 3A). The preferential localization of CK2 α and α' in cytoplasm without DNA damage is due to incubation with Mitotracker (compare with Fig. 3B).

We next determined the time course of changes in the localization of CK2 α and α' in nontransfected HeLa cells over the initial 24 hours following 10 Gy IR treatment (Fig. 3B). The perinuclear localization of CK2 α began at 2 hours and continued to increase to 24 hours, whereas the nuclear localization of CK2 α' was not evident until ~6 hours following IR. These data suggest that CK2 is a relatively late-response protein to IR damage compared with early IR–response proteins, such as 53BP1, which can form nuclear foci within 10 minutes of IR treatment (24).

Whereas IR causes multiple types of IR damage, including DNA double-strand breaks (which are considered the primary lesion leading to cytotoxicity), DNA single-strand breaks, and DNA-protein cross-links, these lesions are typically repaired (or misrepaired) within the first 1 to 2 hours following IR. The observed time course of localization changes of CK2 α and α' from 2 to 6 hours following IR suggests that the participation of CK2 α and α' in IR damage response are temporally related downstream events and that the CK2 catalytic subunits seem to have different cellular targets following IR damage (see Discussion).

**CK2-reduced cells have a normal G2-M checkpoint response after ionizing radiation.** To examine whether CK2 participates in the G2-M arrest following IR, the G2-M checkpoint response in CK2 knockdown HeLa cells was measured by double staining with propidium iodide and mitotic protein monoclonal-2 antibody after nocodazole treatment (Fig. 4). Nocodazole is generally used for a G2-M checkpoint assay (18, 25). IR treatment (30 Gy) strongly inhibits entry into mitosis in control cells and CK2-reduced cells (Fig. 4A, 60% to 4.2% and 38% to 0.2%, respectively), indicating that CK2 is not essential for the G2-M checkpoint. Again, we used Chk1-reduced HeLa cells as a positive control. No inhibition of entry into...
mitosis following IR treatment was found in Chk1 siRNA-treated cells with nocodazole (8.3% and 11%; Fig. 4A). These results indicate that CK2 and Chk1 have different effects on the G2-M checkpoint response in HeLa cells following IR damage.

We previously found a significant delay in cell cycle progression in CK2-reduced cells (Fig. 1D). To determine if this delay was related to IR damage, we used nocodazole to stop the cell cycle at M phase in the absence of IR (Fig. 4B). Cell cycle progression was delayed in both CK2 α– and CK2 α’–reduced cell populations compared with the control. These results suggest that CK2 also participates in cell cycle progression from G1 to M phase, being consistent with a previous report in yeast (3). When CK2 α’ is
reduced, the G₁ population was significantly higher (19% at 24 hours) than in the control cells (2.8% at 24 hours), suggesting preferential involvement of α’ in G₁-S progression. These data are consistent with the results in Fig. 1D. When CK2 α is reduced, the G₂ population was higher (26% at 24 hours) than in α’-reduced cells (13% at 24 hours), suggesting a possible role of CK2 α in G₂-M progression.

A caspase inhibitor inhibits apoptosis following DNA damage. The enhanced apoptotic response in CK2-reduced cells (Figs. 1 and 2) suggests that CK2 controls apoptosis in the presence of IR damage. Therefore, we examined the effect of a general caspase inhibitor, z-VAD. When z-VAD was added to either CK2 α- and α’-reduced cells or Chk1-reduced cells, apoptosis was strongly inhibited after IR treatment, indicating the involvement of caspases (Fig. 5A). The G₂-M population decreased in CK2-reduced cells after IR treatment, suggesting again that CK2 also regulates cell cycle.

Recently, it was reported that ARC could be phosphorylated by CK2 at Thr¹⁴⁹, which regulates the apoptosis inhibitory function of ARC (26). Therefore, we expressed ARC and its mutants, 149A (Thr to Ala, an unphosphorylatable form) and 149E (Thr to Glu, a phosphorylation-mimicking form), in HeLa cells (Fig. 5B). After treatment with CK2 siRNA or Chk1 siRNA (Fig. 5C), the wild-type ARC reduces the extent of apoptosis in both IR-treated cells and nontreated cells (Fig. 5D). However, the mutant ARC transfectants did not show significant differences in the reduction of apoptosis compared with the wild-type ARC transfectants. These results suggest that Thr¹⁴⁹ is not critical for the inhibition of apoptosis induced by IR damage, which differs from the apoptosis mechanism found in a previous study (26).

Discussion

In this study, we show that apoptosis is enhanced following IR by a reduction of the CK2 α and/or α’ subunits through siRNA transfection in HeLa cells (Figs. 1 and 2). A small subset of CK2 α changes localization to perinuclear structures, whereas a marked nuclear accumulation of α’ occurs over 24 hours following IR (Fig. 3). This is the first report indicating dynamic localization changes of the CK2 catalytic subunits after IR. Taken together with our observed apoptosis induction using α and α’ siRNAs (Fig. 2), our results suggest that CK2 α and α’ may have multiple preferential targets involved in inhibition of apoptosis following IR. Additionally, a pan-caspase inhibitor, z-VAD, reduces the apoptotic responses to IR in CK2-reduced cells (Fig. 5A), suggesting that CK2 regulates caspase activity.

We questioned whether ARC could be one of the downstream targets because ARC has a CK2 phosphorylation site that regulates receptor-mediated apoptosis (26). However, both wild-type and mutant ARCs have similar antiapoptotic effects, indicating that point mutations at the phosphorylation site did not change the apoptotic response to IR (Fig. 5D). We conclude that ARC has a nonspecific inhibitory function in IR-mediated apoptosis, which is very different from receptor-mediated apoptosis previously reported (26). Therefore, it is possible that there are other critical targets downstream of CK2 in the IR damage response. We are currently attempting to define these targets.

Because Chk1 is an essential protein involved in both cell survival and in an IR damage response (22, 23), we used a Chk1 siRNA as a positive control. In the G₂-M checkpoint response after IR treatment, the ATR-Chk1 pathway is recognized to be the central pathway, whereas the ATM-Chk2 pathway is a supporting pathway (27, 28). Consistent with previous reports (22, 23), we find that Chk1 siRNA transfection into HeLa cells induces apoptosis even in the absence of IR damage, but also enhances apoptosis following IR treatment similar to the response to CK2 siRNA-transfected HeLa cells (Fig. 2). On the other hand, our data suggest that Chk1, but not CK2, is involved in the G₂-M checkpoint response following IR (Fig. 4A). The caspase inhibitor z-VAD

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**Figure 5.** z-VAD and ARC partially inhibit apoptosis in CK2-reduced cells after IR treatment. A, HeLa cells were transfected with the indicated siRNAs for 30 hours and then cultured for 20 hours. The caspase inhibitor z-VAD (Biomol) or the solvent, DMSO, was added to the medium. Next, cells were irradiated to 30 Gy, cultured for 48 hours, and then analyzed by flow cytometry. B, Myc epitope–tagged ARC, its mutants, and the vector were transfected into HeLa cells. Proteins were analyzed by Western blotting using anti-Myc antibody. C, protocol for initial siRNA transfection (CK2 α + α’ or Chk1 siRNA) followed by plasmid transfection of ARCs or vector for 68 hours and IR (0 or 30 Gy) after 20 hours. Cells were then harvested at 48 hours following IR. D, flow cytometry results of cell populations from the above protocol at 48 hours following IR (0 or 30 Gy).
reduces the extent of apoptosis following IR treatment in both CK2- and Chk1-reduced cells, suggesting involvement of caspases in both pathways (Fig. 5).

CK2 affects the progression of the cell cycle from G1 to M phase even without IR damage (Figs. 1 and 4B). The G2/M population in CK2-reduced cells is also reduced in the presence of z-VAD and IR (Fig. 5A). These data are consistent with a prior study suggesting that CK2 is required for cell cycle progression during G1 and G2 phases in yeast (3). It is also reported that overexpression of CK2α is associated with increased cell proliferation in transformed fibroblasts (29). Our data suggest that α’ contributes to G1-S progression, and that α contributes to G2-M progression at least in part in HeLa cells (Fig. 4B). Because Cdc2 kinase is the central cell cycle regulator whose Ser39 is phosphorylated by CK2 (13), we examined whether a 39A mutation of Cdc2 affects the cell cycle progression in CK2-reduced cells. However, we found no clear differences in the cell cycle distribution between CK2-reduced cells highly expressing wild-type Cdc2 and Cdc2 39A mutants after IR treatment (data not shown).

To examine the initial induction of DNA double-strand breaks by IR and subsequent repair at 30 minutes, we used pulse field gel electrophoresis of chromosomal DNA following 0, 20, and 50 Gy. There was no enhancement in DNA breaks on chromosomes in CK2-reduced cells compared with controls (data not shown). These results suggest that CK2 is not involved in initial double-strand break repair at least under these conditions. This may be reasonable because CK2 has no typical regions related to DNA repair enzymes. We speculate that CK2 can control apoptosis downstream of IR-related DNA repair and its cell cycle effects.

In summary, we show that the CK2 catalytic subunits, α and α’, independently contribute to inhibition of apoptosis after IR damage. This is the first report indicating involvement of CK2 in IR-induced apoptosis and demonstrating a relocalization of the CK2 catalytic subunits within 2 to 6 hours of IR damage.

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