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Activated Checkpoint Kinase 2 Provides a Survival Signal for Tumor Cells

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Abstract

Tumor cells often become resistant to DNA damage–based therapy; however, the underlying mechanisms are not yet understood. Here, we show that tumor cells exposed to DNA damage counteract cell death by releasing the antiapoptotic protein, survivin, from mitochondria. This is independent of p53, and requires activated checkpoint kinase 2 (Chk2), a putative tumor suppressor. Molecular or genetic targeting of Chk2 prevents the release of survivin from mitochondria, enhances DNA damage–induced tumor cell apoptosis, and inhibits the growth of resistant *in vivo* tumors. Therefore, activated Chk2 circumvents its own tumor-suppressive functions by promoting tumor cell survival. Inhibiting Chk2 in combination with DNA-damaging agents may provide a rational approach for treating resistant tumors. (Cancer Res 2006; 66(24): 11576-9)

Introduction

DNA-damaging agents continue to be mainstays of anticancer therapy (1). Checkpoint kinases, Chk1 and Chk2 (2), are modulators of the DNA damage response, and relay signals to execute cell cycle arrest or apoptosis (3). Although Chk2 plays a role in cell cycle control (3, 4), how this pathway couples to cell survival is controversial, especially with respect to p53 (5), and is associated with activation (6–8) or inhibition (9–11) of apoptosis after stress/DNA damage.

Survivin regulates cell division and inhibits apoptosis in cancer (12), and has been implicated as a radioresistance factor in tumor cells (13). This cytoprotective function involves the discharge of a pool of survivin localized to the mitochondria, which inhibits apoptosis and promotes *in vivo* tumor growth (14). Here, we investigated the signaling circuits initiated by DNA damage in tumor cells.

Materials and Methods

Cells and cell cultures. Breast adenocarcinoma MCF-7, colon adenocarcinoma HCT116, or prostate adenocarcinoma PC3 cells were from the American Type Culture Collection (Manassas, VA). Wild-type (WT), p53$^{-/-}$, or Chk2$^{-/-}$ HCT116 cells were kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). HeLa cells were stably transfected with green fluorescent protein (GFP)-survivin targeted to

mitochondria by the cytochrome *c* mitochondrial import sequence, as previously described (14). WT HCT116 cells were stably transfected with a kinase-dead Asp^{347} → Asn Chk2 dominant negative (DN) cDNA (15) under the control of a tetracycline (tet)-regulated promoter (tet-on, Clontech, Palo Alto, CA), with conditional induction by 2 μg/mL of doxycycline.

Modulation of protein expression after DNA damage. Tumor cells were exposed to ionizing radiation (5–10 Gy) using a ^{137}Cs source (Gammacell 40), or treated with Adriamycin (0–100 nmol/L; Sigma), harvested after 2 to 48 hours, and analyzed by Western blotting (14). The following antibodies to survivin (1:1,000; Novus Biologicals) were used: Chk2 (1:500; Upstate Biotechnology), cytochrome *c* (1:100; BD Biosciences-PharMingen), COX-IV (1:500; BD Biosciences-Clontech), p34^{cdc2} (Cdk1; Santa Cruz Biotechnology), XIAP (1:1,000; Transduction Laboratories), or β-actin (1:5,000; Sigma-Aldrich).

Subcellular fractionation. Mitochondrial and cytosolic fractions were prepared as previously described (14).

Transfections. Gene silencing by small interfering RNA (siRNA) was carried out with control (VIII) or survivin-derived (S4) double-stranded RNA oligonucleotide (50 nmol/L), or alternatively, SMART pool siRNA oligonucleotides directed against Chk1 or Chk2 (Dharmacon). A replication-deficient adenovirus expressing Chk2-DN was constructed using the pAd-Easy system, and used at a multiplicity of infection of 50.

Fluorescence microscopy. Analysis of mitochondrially targeted GFP-survivin after ionizing radiation was carried out as previously described (14).

Apoptosis and colony formation assays. Tumor cells treated with or without ionizing radiation were harvested after 24 hours, and analyzed for DNA content by propidium iodide staining, or alternatively, caspase activity (CaspaTag, Intergen) and plasma membrane integrity by multiparametric flow cytometry (14). Colony formation in soft agar of transduced WT HCT116 cells was carried out as described (14).

Xenograft tumor model. All experiments involving animals were approved by an Institutional Animal Care and Use Committee. Two HCT116 clones stably transfected with tet-Chk2-DN, exhibiting sensitivity (no. 102) or resistance (no. 202) to Adriamycin were used. Cells (2.5 × 10^6) were injected into the flanks of 6- to 8-week-old female immunocompromised CB17 severe combined immunodeficiency/beige mice (two tumors/mouse). Upon the appearance of tumors (100–150 mm^3), animals were randomized into groups and administered doxycycline in the drinking water (200 μg/mL in 5% sucrose), with or without Adriamycin (2 mg/kg, i.p. 4 d/wk). Tumor growth was monitored with a caliper according to the formula 1/2 [length (mm)] × [width (mm)]^2, as described in ref. 14. At the end of the experiment, tumors were excised, and frozen sections from the various groups were stained with IgG or a FITC-conjugated antibody to HA, and analyzed by fluorescence microscopy.

Statistical analyses. Data were analyzed using the two-sided unpaired t test on a GraphPad software package for Windows (Prism 4.0). $P = 0.05$ was considered statistically significant.

Results

Regulation of survivin expression by DNA damage. Exposure of MCF-7 cells to ionizing radiation (Fig. 1*A*), or treatment of PC3 cells with Adriamycin (Fig. 1*B*), resulted in time- and
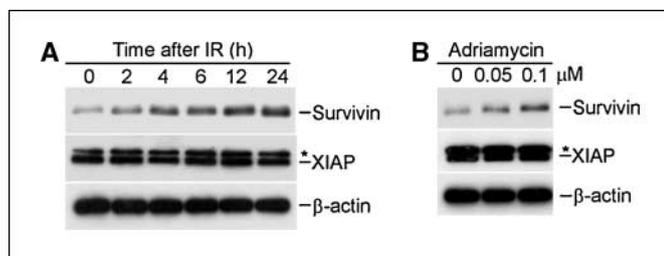

Figure 1. Regulation of survivin expression by DNA damage. *A,* ionizing radiation treatment. MCF-7 cells exposed to ionizing radiation were analyzed by Western blotting. *B,* Adriamycin treatment. PC3 cells treated with Adriamycin were analyzed by Western blotting. *, nonspecific.

concentration-dependent increased expression of survivin. In contrast, DNA damage did not affect the expression of another related protein, XIAP, or β-actin (Fig. 1*A* and *B*). To determine whether Chk1 or Chk2 (2) were involved in this response, we first transfected MCF-7 cells with control vector or a kinase-dead Asp^{347} → Asn Chk2-DN mutant. The expression of Chk2-DN abolished the increase in survivin mediated by ionizing radiation, whereas a control vector was ineffective (Fig. 2*A*). We next ablated Chk2 or Chk1 by siRNA, and analyzed changes in survivin levels after DNA damage. siRNA targeting Chk1 or Chk2 suppressed the expression of the two kinases, respectively, whereas Chk1 siRNA did not affect Chk2, and vice versa (Fig. 2*B*). Without ionizing radiation, no significant modulation of survivin was observed after transfection with the various siRNA (Fig. 2*B*). In contrast, Chk2 knockdown in MCF-7 cells abolished survivin induction by ionizing radiation, whereas Chk1 knockdown or a nontargeted siRNA had no effect (Fig. 2*B*). Finally, ionizing radiation increased survivin expression in a time-dependent manner in Chk2$^{+/+}$ HCT116 cells, but not in Chk2$^{-/-}$ cells (ref. 5; Fig. 2*C*). Chk2$^{-/-}$ cells exhibited constitutively increased expression of endogenous survivin (Fig. 2*C*) and Bcl-2 (Fig. 2*D*), as compared with parental HCT116 cells. This may reflect compensatory changes to increase the antiapoptotic threshold of these cells after loss of Chk2 (see below).

Mechanism of DNA damage–induced survivin expression. Ionizing radiation did not increase *de novo* survivin gene transcription by luciferase promoter analysis, and expression of a p34^{cdc2} Asp146 → Asn kinase-dead mutant that interferes with mitotic progression also had no effect (Supplemental Fig. S1). Ionizing radiation modulates survivin independently of gene expression or the G_2-M checkpoint. Therefore, we asked whether DNA damage induced the redistribution of survivin from existing intracellular pools, and we focused on the fraction of survivin that localizes to the mitochondria (14). Without ionizing radiation, there was no modulation of mitochondrial survivin levels in MCF-7 cells (Fig. 3*A*). Conversely, ionizing radiation induced nearly complete discharge of mitochondrial survivin, which coincided with increased survivin expression in the cytosol, peaking at 12 hours after stimulation (Fig. 3*A*). Consistent with this time course, HeLa cells expressing mitochondrially targeted GFP-survivin exhibited progressive redistribution of the GFP signal from mitochondria to the cytosol in response to ionizing radiation (Fig. 3*B* and *C*). We then asked whether Chk2 was required for mitochondrial discharge of survivin after DNA damage. Exposure of Chk2$^{+/+}$ HCT116 cells to ionizing radiation resulted in the discharge of survivin from mitochondria (Fig. 3*D*). In contrast, ionizing radiation did not release survivin from the mitochondria of Chk2$^{-/-}$ HCT116 cells (Fig. 3*D*). Chk2-dependent release of

mitochondrial survivin occurred indistinguishably in HCT116 cells lacking p53, or deficient in the multidomain proapoptotic protein Bax (Supplemental Fig. S2), two regulators of outer mitochondrial membrane permeability.

Role of activated Chk2 in tumor cell survival after DNA damage. Chk2 knockdown combined with DNA damage increased caspase activity and loss of plasma membrane integrity in HCT116 cells by 2- to 3-fold (Fig. 4*A*). This was associated with the dissipation of mitochondrial membrane potential, and enhanced release of cytochrome *c* (Supplemental Fig. S3), independently of p53 (Supplemental Fig. S4). Survivin knockdown also increased apoptosis after ionizing radiation (Fig. 4*A*), consistent with its role as a Chk2 effector, whereas nontargeted double-stranded RNA oligonucleotide had no effect (Fig. 4*A*). We next asked whether disabling Chk2/survivin cytoprotection affected tumor growth. First, the expression of Chk2-DN or survivin-DN (16) nearly completely abolished HCT116 colony formation in soft agar following ionizing radiation, but not in the absence of ionizing radiation (Fig. 4*B*). Second, HCT116 cells conditionally expressing Chk2-DN after doxycycline induction (Supplemental Fig. S5) exhibited a 2- to 4-fold increase in apoptosis in response to Adriamycin (Fig. 4*C*). Third, we injected tet-Chk2-DN HCT116 clones sensitive (no. 102) or resistant (no. 202) to Adriamycin in immunocompromised mice to form superficial tumors. Adriamycin treatment (2 mg/kg) inhibited the growth of sensitive tumors by 50% to 60% (clone no. 102), but had no effect on resistant tumors (clone no. 202; Fig. 4*D*). In contrast, conditional expression of
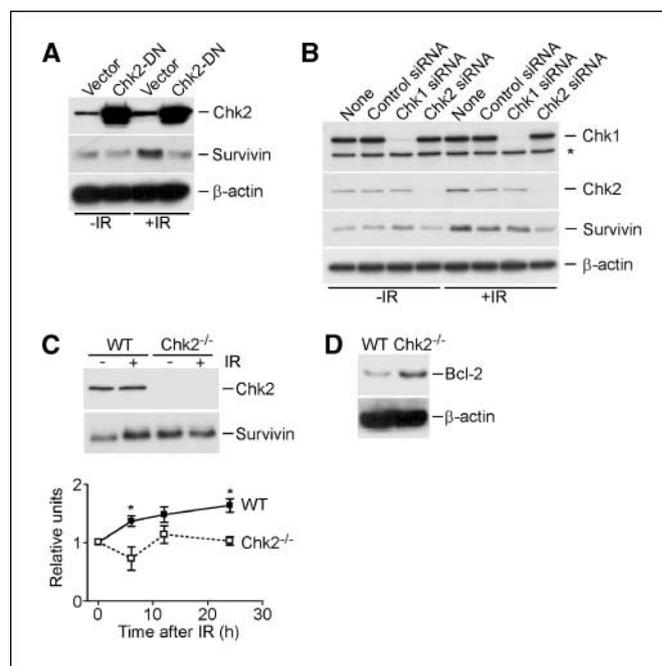

Figure 2. Requirement of Chk2 for modulation of survivin expression after ionizing radiation. *A,* Chk2-DN. Transfected MCF-7 cells with or without ionizing radiation were analyzed by Western blotting. *B,* siRNA knockdown. MCF-7 cells transfected with the indicated siRNA were treated with or without ionizing radiation, and analyzed by Western blotting. *, nonspecific. *C,* Chk2$^{-/-}$ cells. *Top,* WT or Chk2$^{-/-}$ HCT116 cells were treated with or without ionizing radiation, and analyzed by Western blotting. *Bottom,* WT or Chk2$^{-/-}$ HCT116 cells were treated with ionizing radiation, harvested at the indicated time intervals, and analyzed for changes in survivin levels by Western blotting and quantitative densitometry. *Points,* means; *bars,* SE (n = 3); *, P = 0.01 to 0.044. *D,* modulation of Bcl-2 expression. WT or Chk2$^{-/-}$ HCT116 cells were analyzed by Western blotting.
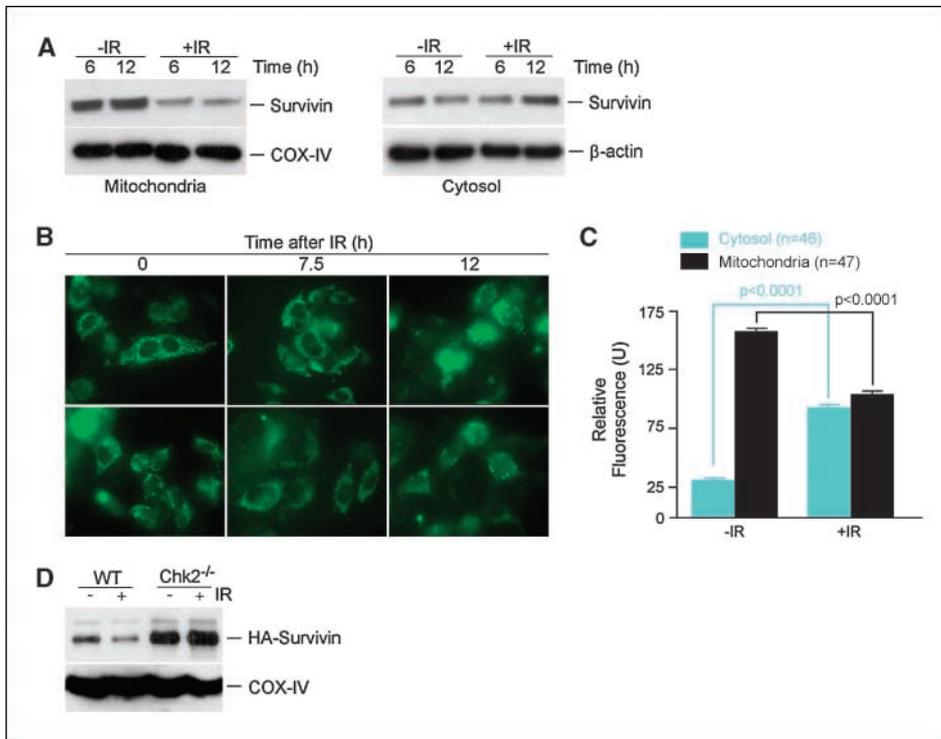
Figure 3. Mechanism of survivin modulation by DNA damage. *A,* mitochondrial release of survivin. Untreated or ionizing radiation–treated MCF-7 cells were fractionated in mitochondrial (*left*) or cytosol (*right*) extracts, and analyzed by Western blotting. *B,* fluorescence analysis. HeLa cells stably transfected with mitochondrially targeted GFP-survivin were treated with ionizing radiation, and analyzed by fluorescence microscopy. Two independent fields per condition (*top/bottom*). *C,* quantification of single cell fluorescence analysis. Individual cytosolic or mitochondrial areas were analyzed for changes in fluorescence intensity before or after ionizing radiation. *Columns,* means; *bars,* SE (n = 46-47). *D,* requirement of Chk2 for mitochondrial release of survivin. Chk2^{+/+} or Chk2^{−/−} HCT116 cells were transduced with adenovirus encoding mitochondrially targeted HA-survivin, treated with or without ionizing radiation, and isolated mitochondrial fractions were analyzed by Western blotting.
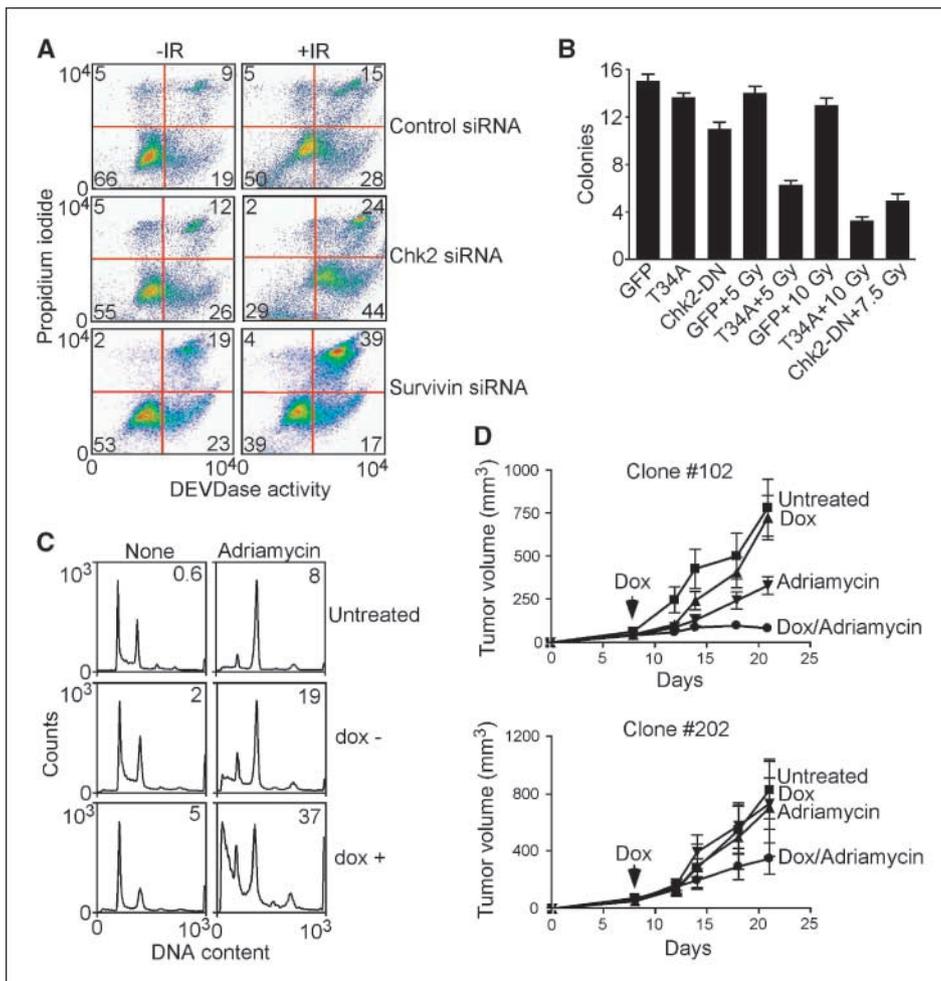

Figure 4. Role of Chk2 cytoprotection in tumor growth. *A,* apoptosis. WT HCT116 cells transfected with the indicated siRNA were treated with or without ionizing radiation, and analyzed for DEVDase activity (caspase activity, *green channel*) and propidium iodide staining (*red channel*). The percentage of cells in each quadrant is indicated. *B,* colony formation assay. WT HCT116 cells expressing the indicated constructs were treated with or without ionizing radiation, and analyzed for colony formation in soft agar. *Columns,* means; *bars,* SD (n = 2). *C,* conditional induction of apoptosis. HCT116 cells (no. 102) transfected with doxycycline-Chk2-DN were treated with or without Adriamycin in the presence or absence of doxycycline (*dox*), and analyzed for DNA content. The percentage of cells with hypodiploid DNA content is indicated. *D,* tumor growth. Adriamycin-sensitive (no. 102) or -resistant (no. 202) HCT116 clones transfected with doxycycline-Chk2-DN were injected s.c. in immunocompromised mice. Animals were administered doxycycline with or without Adriamycin given 4 d/wk. *Points,* means of individual tumor determinations; *bars,* SE (no. 102, n = 14; no. 202, n = 6).

Chk2-DN combined with Adriamycin ablated the growth of sensitive tumors (clone no. 102), and reduced the expansion of resistant tumors (clone no. 202) *in vivo* (Fig. 4*D*). Histologically, tumors treated with the combination of Adriamycin and Chk2-DN exhibited extensive areas of necrosis, as compared with untreated tumors, or animals receiving Adriamycin or doxycycline alone (Supplemental Fig. S6). Tumors from doxycycline-treated animals, but not from untreated mice, stained with an antibody to HA (Supplemental Fig. S7), confirming the conditional expression of recombinant Chk2-DN *in vivo*.

Discussion

In this study, we have shown that activated Chk2 (2) is required to release antiapoptotic survivin from mitochondria (12) following DNA damage. Genetic or molecular targeting of Chk2 prevents survivin release from mitochondria, enhances apoptosis induced by DNA-damaging agents, inhibits colony formation in soft agar, and suppresses tumor growth *in vivo*.

Although considered as a tumor suppressor for its involvement in cell cycle checkpoints (2), these data suggest that Chk2 may play a more complex role in tumor cells, including an unexpected survival signal. The Chk2-regulated discharge of mitochondrial survivin may enhance cell viability during checkpoint activation or oncogene expression, and antagonize mitochondria-dependent apoptosis (14), and mitotic catastrophe (17). How activated Chk2 regulates mitochondrial discharge of survivin remains to be elucidated. However, this pathway may be selectively exploited in transformed cells, as opposed to normal tissues. This may explain why normal tissues exhibit apoptotic defects after Chk2 deletion (6), and Chk2 mutations/deletions have been associated with low penetrance cancer risk (2), whereas Chk2 targeting in tumor cells causes apoptosis (11, 17, 18). Therefore, pharmacologic targeting of Chk2 (19) may be rationally used in combination with DNA damage (1) to enhance apoptosis, especially in resistant tumors. Because Chk2 *directly* couples to mitochondrial cell death independently of cell cycle progression, and mitochondrial survivin is selectively expressed in tumors (14), this strategy may not require the integrity of the G_2 checkpoint, and provide a desirable therapeutic window with limited toxicity for normal tissues.

Acknowledgments

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