Death Receptor-induced Apoptosis Reveals a Novel Interplay between the Chromosomal Passenger Complex and CENP-C during Interphase[□]

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Despite the fact that the chromosomal passenger complex is well known to regulate kinetochore behavior in mitosis, no functional link has yet been established between the complex and kinetochore structure. In addition, remarkably little is known about how the complex targets to centromeres. Here, in a study of caspase-8 activation during death receptor-induced apoptosis in MCF-7 cells, we have found that cleaved caspase-8 rapidly translocates to the nucleus and that this translocation is correlated with loss of the centromere protein (CENP)-C, resulting in extensive disruption of centromeres. Caspase-8 activates cytoplasmic caspase-7, which is likely to be the primary caspase responsible for cleavage of CENP-C and INCENP, a key chromosomal passenger protein. Caspase-mediated cleavage of CENP-C and INCENP results in their mislocalization and the subsequent mislocalization of Aurora B kinase. Our results demonstrate that the chromosomal passenger complex is displaced from centromeres as a result of caspase activation. Furthermore, mutation of the primary caspase cleavage sites of INCENP and CENP-C and expression of noncleavable CENP-C or INCENP prevent the mislocalization of the passenger complex after caspase activation. Our studies provide the first evidence for a functional interplay between the passenger complex and CENP-C.

INTRODUCTION

The kinetochore directs the accurate segregation of chromosomes during cell division. This structure is highly complex, with 60 or more components identified to date in the relatively simple budding yeast centromere (Cheeseman *et al.*, 2002b; Kline-Smith *et al.*, 2005). The first centromeric proteins were identified using autoimmune sera, and they were designated centromere protein (CENP)-A to -C (Earnshaw and Rothfield, 1985). CENP-A is a histone H3 variant (Palmer *et al.*, 1991; Sullivan *et al.*, 1994) that is an essential component at or near the head of the pathway of centromere assembly (Oegema *et al.*, 2001). CENP-B binds to a specific sequence in the α -satellite DNA throughout the centromeric heterochromatin below the kinetochore plate, but it is of unknown function (Masumoto *et al.*, 1989; Cooke *et al.*, 1990). CENP-C, a DNA-binding protein concentrated in the inner kinetochore plate, is downstream of CENP-A, but it is required for the assembly of most other kinetochore components (Saitoh *et al.*, 1992; Cleveland *et al.*, 2003; Maiato *et al.*, 2004). Although generally studied in the context of mitotic

kinetochore function, CENP-A and CENP-C have been shown to be marked for destruction during interphase by the Herpes simplex ICP0 protein (Everett *et al.*, 1999; Lomonte *et al.*, 2001). This cleavage of centromeric components triggers the recently discovered interphase centromere damage response, which involves the functional reorganization of Cajal bodies and Gemini of coiled bodies (GEMs) (Lomonte, personal communication).

The chromosomal passenger complex, which includes INCENP, Aurora B, Survivin, and Borealin/Dasra B, is a key regulator of mitotic events (Cooke *et al.*, 1987; Uren *et al.*, 2000; Gassmann *et al.*, 2004; Vagnarelli and Earnshaw, 2004). The active agent in this complex, Aurora B kinase (Adams *et al.*, 2000), is required for the final stages of chromosome condensation, for chromosomes to achieve a proper bipolar attachment to the spindle, for the function of the spindle assembly checkpoint that monitors this bipolar attachment, and ultimately for the successful completion of cytokinesis (Adams *et al.*, 2001; Carmena and Earnshaw, 2003; Honda *et al.*, 2003; Gassmann *et al.*, 2004; Vagnarelli and Earnshaw, 2004). Aurora B phosphorylates numerous chromosomal substrates, including histone H3 (Hsu *et al.*, 2000; Murnion *et al.*, 2001); CENP-A (Zeitlin *et al.*, 2001b); the regulator of centromeric cohesion MEI-S332 (Resnick *et al.*, 2006); the mitotic centromere-associated kinesin (MCAK) (Andrews *et al.*, 2004; Lan *et al.*, 2004); and Hec1/Ndc80, a kinetochore component essential for microtubule binding (Cheeseman *et al.*, 2006; DeLuca *et al.*, 2006). Phosphorylation of Hec1/Ndc80 is essential for the correction of attachment errors during mitosis.

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Recent results reveal that INCENP is a nexus for mitotic kinase signaling, as in addition to its binding of Aurora B, it also binds to Plk1 and directs the targeting of this essential kinase to kinetochores (Goto *et al.*, 2005; Carmena and Earnshaw, 2006). Despite its demonstrated role in regulation of kinetochore attachment to microtubules (Tanaka *et al.*, 2002), RNA interference (RNAi) studies suggested that targeting of kinetochore components such as CENP-A and the chromosomal passenger proteins were not functionally linked (Oegema *et al.*, 2001), and little is known about how the passengers are targeted to centromeres (Vader *et al.*, 2006).

Apoptosis is a major form of cell death, used to remove unwanted or excess cells. Two predominant pathways of apoptosis have been described: the intrinsic pathway, which involves initial mitochondrial perturbation resulting from cellular stress or cytotoxic insults, and the extrinsic pathway, which is triggered by activation of death receptors of the tumor necrosis factor (TNF) family (Earnshaw *et al.*, 1999; Bratton *et al.*, 2000). Caspase-8 and -9 are the apical caspases in the extrinsic and intrinsic pathways, respectively, and they activate the effector caspases, -3, -6, and -7, which cleave many cellular substrates, resulting in the characteristic morphological and biochemical changes of apoptosis (Earnshaw *et al.*, 1999; Sun *et al.*, 1999). Ligation of the death receptors, such as CD95 (Fas/Apo1), TNF receptor (TNF-R)1, and the TNF-related apoptosis-inducing ligand (TRAIL) receptors, by their cognate ligands or agonistic antibodies results in receptor aggregation and recruitment of the adaptor protein MORT1/Fas-associated death domain (FADD) (Ashkenazi and Dixit, 1998; Wallach *et al.*, 1999). FADD then recruits the initiator caspase-8, which is activated within the death-inducing signaling complex (for review, see Peter and Krammer, 2003). However, recent studies have shown that caspase-8 and FADD are not recruited to a TNF-induced membrane-bound receptor signaling complex but instead are activated elsewhere within the cell (Harper *et al.*, 2003; Micheau and Tschopp, 2003).

The present study was instigated to ascertain where caspase-8 is activated after TNF stimulation. Surprisingly, in response to TNF or TRAIL, we observed an early localization of active caspase-8 in the nucleus accompanied by a loss of CENP-C and INCENP. Mapping of the cleavage sites and construction of noncleavable mutants have revealed for the first time that localization of the chromosomal passenger complex to kinetochores requires intact CENP-C, whereas conversely, localization of CENP-C to kinetochores requires intact INCENP. This is the first demonstration of a functional interplay between the chromosomal passenger complex and an integral kinetochore component.

MATERIALS AND METHODS

Materials

Media and serum were from Invitrogen (Paisley, United Kingdom). The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD.fmk) was from Enzyme Systems (Dublin, CA). [³⁵S]methionine was from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Recombinant TRAIL and active caspase-3, -6, -7, and -8 were prepared as described previously (MacFarlane *et al.*, 1997; Sun *et al.*, 2004), and caspase activity was determined by active site titration with z-VAD.fmk (Stennicke and Salvesen, 1999). All other chemicals were purchased from Sigma Chemical (Poole, Dorset, United Kingdom) or Fisher (Loughborough, Leicestershire, United Kingdom).

Cell Culture, Transient Transfection, and Cell Synchronization

MCF-7-Fas (MCF-7) human breast epithelial cells (from M. Jattella, Danish Cancer Society Research Center, Copenhagen, Denmark); MCF-7-Bcl-x_L overexpressing cells (from K. Schulze-Osthoff, University of Dusseldorf, Dussel-

dorf, Germany); wild-type Jurkat E6.1 (European Collection of Animal Cell Cultures, Porton Down, Wiltshire, United Kingdom) FADD-deficient and caspase-8-deficient Jurkat cells (from J. Blenis, Harvard Medical School, Boston, MA), and c-FLIPs overexpressing Jurkat cells (from J. Tschopp, University of Lausanne, Epalinges, Switzerland) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM GlutaMAX (Invitrogen). A549 human lung type II pneumocytes and HeLa cells (European Collection of Animal Cell Cultures) were grown in DMEM supplemented with 10% FBS. Cells for transfection were plated on coverslips in 12-well plates and transiently transfected with FuGENE 6 (Roche Diagnostics, Mannheim, Germany). After 24 h, cells were exposed to various apoptotic stimuli as described. Cells were synchronized using thymidine and nocodazole as described in figure legends.

Antibodies

The following antibodies were used for Western blotting and/or immunofluorescence: cleaved caspase-8 (Cell Signaling Technology, Beverly, MA); anti-phospho-histone H3 (Ser10) (Upstate Biotechnology, Lake Placid, NY); Aurora B and caspase-6 (BD Transduction Laboratories, Lexington, KY, and Abcam, Cambridge, United Kingdom); hemagglutinin (HA)-tag (Roche Diagnostics); BubR1 antibody (Ditchfield *et al.*, 2003); provided by S. Taylor, University of Manchester, Manchester, United Kingdom); c-myc antibody (Cell Signaling Technology, Beverly, MA); and CENP-C, INCENP, Survivin, and Borealin (Saitoh *et al.*, 1992; Adams *et al.*, 2001; Ditchfield *et al.*, 2003). The following antibodies were used for Western blotting: T7 (Merck Biosciences, Darmstadt, Germany), anti-CENP-C (provided by K. Yoda, Nagoya University, Nagoya, Japan; Ando *et al.*, 2002), and caspase-7 and -8 antibodies that recognize the intact zymogen and the processed forms (Sun *et al.*, 1999). The CENP-A antibody for immunofluorescence was provided by M. Valdivia (University of Cádiz, Cádiz, Spain) (Valdivia *et al.*, 1998). Anti-centromere (ACA), anti-CENP-B, and PIKA antibodies were used for immunofluorescence (Saunders *et al.*, 1991; Saitoh *et al.*, 1992).

Immunofluorescence Microscopy

Cells were grown on coverslips, and after treatment they were washed with phosphate-buffered saline (PBS), fixed using 3.8% paraformaldehyde for 15 min, rinsed in PBS, and permeabilized in 0.2% Triton X-100 in 3% bovine serum albumin (BSA) for 5 min. After rinsing in PBS, blocking with 3% BSA for 30 min, and rewashing in PBS, the cells were incubated in primary antibodies diluted in 3% BSA for 60 min at room temperature. The cells were then washed in PBS and incubated for 60 min with anti-Alexa Fluor 568 and/or 488 antibodies (Invitrogen) diluted in 3% BSA. After washing with PBS, the nuclei were stained by incubating the cells for 10 min with 250 ng ml⁻¹ Hoechst 33258 (Calbiochem, San Diego, CA) at room temperature. The coverslips were washed with PBS and then mounted onto slides using Vectashield (Vector Laboratories, Burlingame, CA). Optical sections were taken using an argon-krypton laser and either a TCS-4D (Leica, Wetzlar, Germany) or LSM 510 Axiovert 200 (Carl Zeiss, Jena, Germany) confocal imaging system. Microscopes were equipped with either a 63× numerical aperture (NA) 1.4 or a 100× NA 1.4–0.7 (Leica) oil immersion objectives and standard filter sets. Images were cropped in Adobe Photoshop 5.5 (Adobe System, Mountain View, CA) and were sized and placed using Adobe Illustrator CS2 (Adobe Systems). The cell counts represent the median of at least three separate experiments in which at least 500 (Figures 2–4) or 100 (Figures 5 and 6) were counted per treatment per experiment.

Western Blot Analysis

Protein samples obtained from equal numbers of cells or nuclei were lysed in SDS-PAGE loading buffer and separated by electrophoresis on SDS-polyacrylamide gels appropriate to the molecular weight of the protein of interest, followed by electrophoretic transfer onto nitrocellulose (Hybond-C Extra; GE Healthcare) and detection as described previously (Sun *et al.*, 1999; Sun *et al.*, 2004).

RNA Interference

MCF-7 cells were resuspended to a concentration of 10,000 cells/well in 12-well plates and transfected with small interfering RNA (siRNA) oligonucleotides at a final concentration of 100 nM by using Effectene (QIAGEN, Dorking, Surrey, United Kingdom). After 24 h, cells were retransfected and 24 h later, they were exposed to apoptotic stimuli and processed for SDS-PAGE analysis or immunofluorescence. Caspase-7 oligonucleotides were from Ambion (Silencer Pre-designed siRNAs) and XIAP and scrambled oligonucleotides were from Dharmacon RNA Technologies (Lafayette, CO).

Generation of Protein Expression Constructs

Full-length CENP-C (residues 1–943) (Pluta and Earnshaw, 1996; Yang *et al.*, 1996) was subcloned into pET21b (Novagen, Madison, WI), and pcDNA3.1/Myc-His (Invitrogen) in frame with the C-terminal c-myc tag and full-length INCENP (Honda *et al.*, 2003) was subcloned into pcDNA3.1 HA tag in frame with the C-terminal HA tag by using standard polymerase chain reaction (PCR)-based subcloning techniques. Mutant CENP-C (D624A) and INCENP

(D438A) were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). ^{35}S -labeled proteins were produced using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Custom-designed PCR primers were from Invitrogen.

Cell Fractionation

Cells were trypsinized and washed once in cold PBS (4°C) and once in nuclei preparation buffer containing 250 mM sucrose, 20 mM HEPES-NaOH, pH 7.4, 0.15 mM spermine and 0.5 mM spermidine, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Roche Diagnostics). Cells were resuspended in this buffer containing 0.1% digitonin, kept on ice for 30 min, and then passed through a 25-gauge needle 20 times before centrifugation at 2000 rpm at 4°C for 3 min. The resultant nuclei were washed three times with the nuclei preparation buffer (without digitonin). Nuclear preparations ($2 \times 10^6 \text{ ml}^{-1}$) were treated with caspases for 60 min, and the reactions were terminated with equal volumes of 2× SDS loading buffer. Samples for immunofluorescence were removed before the addition of SDS buffer and centrifuged onto glass slides at 600 rpm for 5 min.

Statistical Analysis

Nonparametric methods were used to analyze the data sets. For comparison of two treatment groups, Student's *t* test or the Mann-Whitney test was used. The Kruskal-Wallis one-way analysis of variance test was used for comparison of three or more treatment groups. Dunn's test was then used for post hoc comparison between groups (Siegel and Castellan, 1988). Analysis of the data was one tailed, because only decrease in the experimental outcomes/measures was possible. Spearman's rank test was used for testing correlation. A significance level of $p < 0.05$ was used except in the figures where * represents <0.05 , ** represents <0.01 , and *** represents <0.001 .

RESULTS

Processed Caspase-8 Is Found in the Nucleus Shortly after Death Receptor Stimulation

To ascertain where caspase-8 is activated in TNF-induced apoptosis, we used a monoclonal antibody that detects the fully processed p10 subunit of caspase-8 resulting from cleavage at Asp 384, which throughout this study we refer to as cleaved caspase-8. Using this antibody, we detected discrete punctate foci of $0.3\text{--}0.5 \mu\text{m}$ within the nuclei of MCF-7 cells within 30 min of exposure to TNF/cycloheximide (Figure 1A) whereas no such labeling was observed in untreated cells (Figure 1A). Similar activation occurred with TRAIL, another TNF family member, when cleaved caspase-8 was seen within the nucleus of some cells as early as 5 min posttreatment (Figure 1A) and was retained in the nucleus up to 30 min. At later times (60 min), cleaved caspase-8 was also seen in the cytoplasm (Figure 1A). The appearance of cleaved caspase-8 in the nucleus at 5 min detected by confocal microscopy preceded the detection of processed caspase-8 (15 min) by Western blot analysis (Supplemental Figure 1).

To determine whether cleaved caspase-8 accumulated in the nuclei of cells other than MCF-7 cells, which are deficient in caspase-3 (Janicke *et al.*, 1998), HeLa and A549 cells were exposed to TRAIL. Cleaved caspase-8 was initially observed in the nucleus and was seen in the cytoplasm at later times in both cell lines (Figure 1B). Nuclear cleaved caspase-8 was also present in wild-type Jurkat cells exposed to TRAIL, but it was absent in caspase-8-deficient, FADD-deficient, and c-FLIP_s-overexpressing Jurkat cells (Figure 1C). These results further support the specificity of the nuclear caspase-8 labeling. Induction of apoptosis by TRAIL resulted in a time-dependent processing of caspase-8 initially to its p43- and p41-processed forms in wild-type but not in FADD-deficient or c-FLIP_s-overexpressing Jurkat cells (Figure 1D, top three boxes). Using the same neopeptide antibody as in the immunofluorescence studies, a time-dependent appearance of the p10 catalytically active small subunit of caspase-8 was also detected after exposure to TRAIL (Figure 1D, bottom box). The appearance of cleaved caspase-8 within the

Figure 1. Rapid translocation of cleaved caspase-8 to the nucleus in death receptor induced apoptosis. In A–C, cells were stained with anti-cleaved caspase-8 (green) and Hoechst 33258 (blue) and analyzed by confocal microscopy. (A) MCF-7 cells were incubated either alone, with recombinant 400 ng ml^{-1} TNF/ $1 \mu\text{M}$ cycloheximide (CHX), or $1 \mu\text{g ml}^{-1}$ TRAIL. Bar, $10 \mu\text{m}$. (B) HeLa or A549 cells were incubated for 60 min with $1 \mu\text{g ml}^{-1}$ TRAIL. (C) Wild-type Jurkat E6.1 cells, caspase-8-deficient, FADD-deficient, or c-FLIP_s-overexpressing Jurkat cells were incubated with $1 \mu\text{g ml}^{-1}$ TRAIL for 1 h. Bar, $10 \mu\text{m}$. (D) Processing of caspase-8 was examined by Western blotting after $1 \mu\text{g ml}^{-1}$ TRAIL treatment. The cleaved caspase-8 antibody was used to detect the appearance of the p10 subunit, whereas a second caspase-8 antibody was used to detect the intact zymogen and the p43/41 processed forms (Sun *et al.*, 1999).

nucleus after induction of apoptosis raised the possibility that caspase-8 was already present within the nucleus before TRAIL treatment, as recently proposed (Besnault-Mascard *et*

Figure 2. Rapid loss of CENP-C from the centromere after the appearance of cleaved caspase-8 within the nucleus. MCF-7 cells were either untreated (A) or treated with $1 \mu\text{g ml}^{-1}$ TRAIL for the indicated times either alone (B or C) or after pre-incubation for 60 min with $10 \mu\text{M}$ z-VAD.fmk (D). Cells were then labeled with antibodies against CENP-C (554) (red), cleaved caspase-8 (green) and stained with Hoechst 33258 (blue). Some cells retaining CENP-C label are marked with an asterisk (A–D). Bar, $15 \mu\text{m}$. (E) Jurkat cells were treated with $1 \mu\text{g ml}^{-1}$ TRAIL for 1 h and similarly labeled. The arrows indicate cells without CENP-C label. Bar, $10 \mu\text{m}$. (F) MCF-7 cells were either untreated (F) or exposed to $1 \mu\text{g ml}^{-1}$ TRAIL (G) and labeled with a different CENP-C antibody (420). Bar, $15 \mu\text{m}$. (H–J) MCF-7 cells were exposed to $1 \mu\text{g ml}^{-1}$ TRAIL as described above but labeled with an ACA antibody against centromeres (red). The TRAIL-induced reduction in ACA staining was prevented by z-VAD.fmk. Bar, $8 \mu\text{m}$. (K) MCF-7 and (L) A549 cells were exposed to TRAIL, and cells were scored for loss of CENP-A, -B, or -C as indicated ($n \geq 3$). For CENP-C, the Kruskal–Wallis test was significant at $p < 0.01$ ($H = 17.72$, $df = 3$). Post hoc comparison against the control showed a significant decrease in median values at 2 and 3 h post treatment ($p < 0.01$). For CENP-A, Kruskal–Wallis was significant at $p < 0.05$ ($H = 9.25$; $df = 3$). A decrease in the median percentage was only seen at 3 h ($p < 0.05$). (L) For CENP-C, the Kruskal–Wallis test was significant at

et al., 2005). However using confocal microscopy, cell fractionation, and Western blotting, we have been unable to demonstrate the presence of either unprocessed or sumoylated caspase-8 within the nucleus (our unpublished data). The failure to detect nuclear cleaved caspase-8 by Western blot analysis at early times after TRAIL treatment could be due both to the lower sensitivity of the method compared with confocal analysis and to the possible leakage of the cleaved caspase from the nuclei after isolation, given the known alterations in the nuclear transport systems early in apoptosis (Ferrando-May, 2005). Therefore, cleaved caspase-8 may be transported to the nucleus after its formation within the cytosol. In support of this, active caspase-8, added to permeabilized MCF-7 cells, was transported into the nucleus, and this transport was blocked by pretreatment with wheat germ agglutinin (Supplemental Figure 2), which inhibits nuclear localization signal-mediated nuclear transport (Newmeyer and Forbes, 1988). Together, these data demonstrate for the first time the rapid appearance of cleaved caspase-8 within the nuclei of various cell types after death receptor stimulation.

Nuclear Cleaved Caspase-8 Precedes a Rapid Loss of CENP-C and Disruption of Centromeres

A striking time-dependent loss of CENP-C labeling was observed in TRAIL-treated cells that contained nuclear cleaved caspase-8, whereas normal centromeric labeling of CENP-C was retained in cells lacking nuclear cleaved caspase-8 (Figure 2, A–C). After 30-min exposure, nuclei containing both cleaved caspase-8 and CENP-C were observed, although the CENP-C labeling was clearly diminished compared with cells not containing cleaved caspase-8 (Figure 2B). After 1-h exposure to TRAIL, cells with nuclear cleaved caspase-8 lost all detectable CENP-C labeling (Figure 2C). Pretreatment with z-VAD.fmk, a broad-spectrum caspase inhibitor, prevented the appearance of cleaved caspase-8 and the loss of CENP-C labeling (Figure 2D), supporting the suggestion that cleaved caspase-8 within the nucleus led either directly or indirectly to the loss of CENP-C labeling. Loss of CENP-C was also observed after TRAIL treatment of Jurkat and A549 cells, showing that this loss is not cell type specific (Figure 2, E and L). To confirm that the TRAIL-induced loss of CENP-C was not due to a specific property of the antibody used (Ab 554), two additional CENP-C antibodies were used. Loss of CENP-C was again observed (Figure 2, F and G; data not shown). No colocalization of cleaved caspase-8 was observed with centromeres, promyelocytic leukemia or polymorphic interphase karyosomal association bodies, after exposure to TRAIL for 1 h (Supplemental Figure 3) (Saunders *et al.*, 1991; Lamond and Earnshaw, 1998; Platani and Lamond, 2004); however, any association of a caspase with its substrate would probably be transient.

To determine whether caspase activation also resulted in loss of other centromeric proteins, we used ACAs, which detect CENP-A, -B, and -C as well as anti-CENP-A and anti-CENP-B antibodies. A clear diminution in ACA staining was observed in cells containing nuclear-cleaved caspase-8, which was also prevented by z-VAD.fmk (Figure 2, H–J), confirming the caspase-dependent change in centromeric structure. MCF-7 cells showed a marked time-dependent loss of CENP-C

$p < 0.01$ ($H = 14.3$, $df = 3$). A significant decrease was apparent at 3 h ($p < 0.01$). For CENP-B, there was no evidence of a significant decrease in medians at any time ($H = 5.6$, $df = 3$).

labeling with a significant decrease in median values at 2 and 3 h after exposure to TRAIL, which preceded and was more extensive than the loss of CENP-A staining (Figure 2K). We were unable to assess the effects of TRAIL on CENP-B in MCF-7 cells, because the antibody did not uniformly label untreated cells. However, in A549 cells, where this was not a problem, extensive loss of CENP-C was again observed after TRAIL treatment without significant loss of CENP-B (Figure 2L). Thus, our data suggest an early relatively selective loss of CENP-C from the centromeres, which precedes a more general disruption of kinetochore chromatin with loss of CENP-A.

Efficient Cleavage of CENP-C by Caspase-7

Our results suggested that caspase-8 could be directly or indirectly responsible for the cleavage and resultant loss of CENP-C from the centromeres. Because direct cleavage of CENP-C by caspase-8 was the simplest hypothesis, we generated full-length ³⁵S-labeled CENP-C protein by in vitro translation (IVT) and exposed it to active recombinant caspases. High concentrations of caspase-8 were required to cleave CENP-C (Figure 3A), suggesting that caspase-8 may promote CENP-C cleavage by activating another caspase. To test this hypothesis, CENP-C was exposed to caspase-6 and -7, the major effector caspases in MCF-7 cells as well as to active caspase-3, used as a positive control. All the caspases cleaved CENP-C in a concentration-dependent manner, albeit with differing efficiencies (Figure 3A). Caspase-7 was clearly the most efficacious, with low concentrations (1–3 nM) resulting in efficient cleavage of CENP-C to an ~80-kDa fragment, and higher concentrations resulting in complete loss of the full-length protein with formation of multiple cleavage products (Figure 3A). Because these results were obtained solely with in vitro recombinant proteins, we examined whether CENP-C cleavage also occurred in intact cells. In MCF-7 cells exposed to TRAIL, little if any cleavage of CENP-C was observed by Western blot analysis, possibly because of the very low abundance of this protein and/or its degradation by the proteasome (Everett *et al.*, 1999). To facilitate study of endogenous CENP-C, isolated nuclei were incubated with recombinant caspases. Caspase-7 (10 nM) and caspase-3 (50 nM) again cleaved endogenous CENP-C to an ~80-kDa fragment, whereas caspase-6 and -8 (200 nM) failed to cleave CENP-C in isolated nuclei (Figure 3B). Using confocal microscopy, loss of kinetochore-associated CENP-C was observed in nuclei exposed to active caspase-7 (10 and 200 nM) as well as caspase-3 (200 nM) but not caspase-6 or -8 (200 nM) (Figure 3, C–H). These results are consistent with specific disruption of centromeres by active caspases, in particular by caspase-7 and caspase-3 with the former being ~5- to 10-fold more efficient at cleaving CENP-C.

These results suggested that caspase-7, rather than caspase-8, might be the primary caspase responsible for CENP-C cleavage. To test this hypothesis, staurosporine was used to induce apoptosis in MCF-7 cells, because this should result in activation of the intrinsic pathway with initial activation of caspase-9, which can then subsequently activate caspase-7 (Slee *et al.*, 1999; Bratton *et al.*, 2001). Caspase-8 should not be activated in this model system, because caspase-3 is required for activation of caspase-8 via caspase-6 downstream of mitochondria (Slee *et al.*, 1999). In support of this prediction, caspase-7 was processed to its catalytically active p19 large subunit without any detectable cleavage/processing of caspase-8 in staurosporine-treated MCF-7 cells (Figure 3I). Staurosporine induced a highly significant time-dependent loss of cells labeled with CENP-C (Figure 3J), further supporting an active role for caspase-7 rather

Figure 3. Efficient cleavage of CENP-C by caspase-7. (A) ³⁵S-labeled CENP-C was incubated for 1 h with active caspases and the cleavage fragments indicated with arrows. (B) Isolated nuclei from MCF-7 cells were incubated for 1 h with active caspases (1–200 nM). Caspase-7 (C7) efficiently cleaved endogenous nuclear CENP-C, detected using a CENP-C antibody. Using confocal microscopy, no loss of kinetochore-associated CENP-C was observed in untreated nuclei (C), but loss was observed when isolated nuclei were incubated for 1 h with caspase-7 (D and E) or caspase-3 (H) but not with either caspase-6 or -8-treated nuclei (F and G). MCF-7 cells incubated with 1 μ M staurosporine showed both (I) activation of caspase-7 but not caspase-8 and (J) loss of CENP-C labeling, assessed by confocal microscopy ($n = 3$). MCF-7 cells showed a highly significant decrease in CENP-C labeling by using Spearman's rank test. From 4 to 6 h, the approximate rate of decrease was $\sim 15\% \text{ h}^{-1}$.

than caspase-8 in CENP-C cleavage. Pretreatment of MCF-7 cells with 50 μ M z-VAD.fmk prevented the staurosporine-induced loss of CENP-C labeling, supporting the suggestion that the loss of CENP-C labeling was due to caspase activation rather than inhibition of protein kinases (Supplemental Figure 4). To further assess the role of caspase-7 in CENP-C cleavage in MCF-7 cells, we depleted caspase-7 by using siRNA. Extensive knockdown of endogenous caspase-7 (75–90%) was observed without any depletion of caspase-8 or -6 (Supplemental Figure 5A).

Knockdown of caspase-7 in Bcl-x_L-overexpressing MCF-7 cells resulted in a significant inhibition of the loss of CENP-

C-labeled cells after exposure to TRAIL (Supplemental Figure 5B), further supporting an important role for caspase-7 in mediating CENP-C loss, at least in MCF-7 cells.

Centromere Disruption Alters Chromosomal Passenger Protein Localization

The activity of the kinetochore in both microtubule transactions and cell cycle signaling is regulated by protein kinases, including Bub1, BubR1, and the chromosomal passenger complex (Cleveland *et al.*, 2003; Vagnarelli and Earnshaw, 2004). In preliminary experiments, no change was observed in BubR1 associated with the kinetochore in TRAIL-treated cells (our unpublished data). However, TRAIL treatment had a profound effect on the localization of the chromosomal passenger proteins. In control HeLa cells at G₂, Aurora B was present in the nucleus and highly concentrated at the centromeres, as shown by its partial colocalization with both CENP-C and CENP-A (Figure 4, A and B). After exposure to TRAIL, Aurora B no longer localized to centromeres (Figure 4, D and E). Strikingly, all cells that had lost CENP-C failed to concentrate Aurora B at the centromeres (Figure 4J). In untreated cells Aurora B largely colocalized with Borealin (Figure 4C). After exposure to TRAIL, Borealin also exhibited a diffuse nuclear staining and was no longer concentrated at the centromeres (Figure 4F). These data reveal that loss of CENP-C correlates with the mislocalization of chromosomal passenger proteins during interphase.

Surprisingly, CENP-C cleavage is required for TRAIL-induced mislocalization of chromosomal passenger proteins. Site-directed mutagenesis revealed DEAD⁶²⁴↓L as the primary caspase-7-cleavage site in CENP-C (Figure 4G). This D(I)XAD motif is conserved in mouse, rat and human. Transfected wild-type and noncleavable CENP-C localized adjacent to Aurora B, showing a normal centromeric distribution (Figure 4, H and I). Furthermore, transfection of the noncleavable but not the wild-type CENP-C prevented the mislocalization of Aurora B compared with control green fluorescent protein (GFP)-transfected cells after exposure to TRAIL (Figure 4K). The ability of noncleavable CENP-C to block Aurora B mislocalization was surprising as CENP-C and the passenger proteins have been shown to target to centromeres independently of one another (Oegema *et al.*, 2001).

INCENP Is Cleaved in TRAIL-treated Cells

Disruption of any one of the chromosomal passenger components prevents localization of the entire complex to centromeres (Adams *et al.*, 2001; Honda *et al.*, 2003; Gassmann *et al.*, 2004). We wanted to determine whether mislocalization of Aurora B after exposure to TRAIL (Figure 4) was due solely to cleavage of CENP-C or whether it might be also due to cleavage of chromosomal passenger proteins. Analysis of synchronized MCF-7 cells in G₂ exposed to TRAIL revealed that INCENP but not Aurora B, Survivin, or Borealin was cleaved by caspases during apoptosis (Figure 5A). Thus, INCENP cleavage could explain the mislocalization of Aurora B in these cells. ³⁵S-labeled INCENP was cleaved by caspase-3, -7, and -8 into two fragments of ~65 kDa, with caspase-7 again being the most effective (Figure 5B). Site-directed mutagenesis revealed DQAD⁴³⁸↓G as the primary caspase-7 cleavage site in INCENP (Figure 5C). This DQXD↓G motif is conserved in human, mouse, and rat. Transfected wild-type and noncleavable INCENP largely colocalized with Aurora B, showing a normal centromeric distribution (Figure 5D, left). After exposure to TRAIL, colocalization of INCENP and Aurora B was disrupted, and this disruption was prevented by transfection of noncleavable but not by

Figure 4. Aurora B mislocalization in cells that have lost CENP-C. HeLa cells were untreated (A–C) or exposed to 1 $\mu\text{g ml}^{-1}$ TRAIL for 1.5 h (D–F). Cells were labeled with anti-Aurora B (green) and anti-CENP-C (red; A and D), anti-CENP-A (red; B and E) or anti-Borealin antibodies (red; C and F). Bar, 8 μm . (G) Wild-type CENP-C and the noncleavable mutant were incubated for 1 h with active 5 nM caspase-7, and samples were analyzed with an anti-T7 antibody. (H–I) HeLa cells were transfected with myc tagged wild type and CENP-C-D624A and labeled with anti-myc (red) and anti-Aurora-B (green) antibodies. Bar, 8 μm . (J) HeLa cells were exposed to 1 $\mu\text{g ml}^{-1}$ TRAIL for 1.5 h and labeled with antibodies against CENP-C and Aurora B. Aurora B mislocalization was counted in cells with cleaved and mislocalized CENP-C. (K) HeLa cells were transfected with GFP control, myc-CENP-C-WT, or myc-CENP-C-D624A and exposed to 1 $\mu\text{g ml}^{-1}$ TRAIL for 1.5 h. Aurora B mislocalization was scored in transfected cells. There was a significant change in the median percentage of Aurora B mislocalization after TRAIL exposure ($H = 7.42$, $df = 3$, $p < 0.025$). Post hoc testing revealed a significant difference between GFP control and the noncleavable mutant ($p < 0.025$) but not wild type. The difference between the wild type and mutant was marginally significant at $p < 0.1$, which in this experiment is evidence of the mutation providing a degree of protection.

wild-type INCENP (Figure 5D, right, and E). These results suggest that caspase-mediated cleavage of INCENP causes the mislocalization of chromosomal passenger proteins. To

Figure 5. Caspase-mediated cleavage of INCENP prevents Aurora B localization to the centromeres. (A) MCF-7 cells were synchronized into S phase for 48 h with 2 mM thymidine. After release into G₂, cells were exposed to 1 μ g ml⁻¹ TRAIL alone or in the presence of 10 μ M z-VAD.fmk (Z) and analyzed by Western blotting with the indicated antibodies. AS, asynchronous cells. (B) ³⁵S-labeled INCENP was incubated for 1 h with active caspases and two cleavage fragments were generated. (C) ³⁵S-labeled wild-type INCENP and a noncleavable mutant (D438A) were incubated for 1 h with active 5 nM caspase-7. (D) HeLa cells were transfected with wild-type and noncleavable D438A HA-tagged INCENP, exposed to 1 μ g ml⁻¹ TRAIL, and labeled with antibodies against HA tag (green) and Aurora B (red). Bar, 10 μ m. (E) HeLa cells were transfected with GFP control, wild-type INCENP, or INCENP-D-A. After 24 h, cells were exposed to 1 μ g ml⁻¹ TRAIL and labeled with antibodies against Aurora B. This treatment caused a significant difference in the median percentage of Aurora B mislocalization ($H = 6.55$, $df = 3$, $p < 0.05$). Post hoc testing showed a significant difference between GFP control and the noncleavable INCENP-D-A mutant ($p < 0.05$) but not with wild-type INCENP-expressing cells. Post hoc testing did not yield a significant difference between the medians of wild-type INCENP and its D-A mutant ($p < 0.05$). (F) HeLa cells were treated with 50 ng ml⁻¹ nocodazole for 16 h, arrested in M phase, and then exposed to 1 μ g ml⁻¹ TRAIL either alone or in the presence of 10 μ M z-VAD.fmk and then subjected to Western blotting.

gain some insight of the functional consequences of this cleavage, HeLa cells, arrested in M phase, were exposed to TRAIL. This resulted in a loss of phosphorylated histone H3 Ser10 that was prevented by z-VAD.fmk (Figure 5F), suggesting that INCENP cleavage may prevent Aurora B activation.

Overexpression of Noncleavable INCENP Prevents Loss of Centromeric CENP-C

Our results demonstrating that expression of noncleavable CENP-C or INCENP prevented the mislocalization of the passenger complex after caspase activation were surprising, because they suggested a previously unknown functional dependence between the passenger complex and CENP-C. To investigate this potential interaction, we expressed both wild-type and noncleavable INCENP and examined their effects both on the potential cleavage of INCENP and CENP-C and also on the loss of CENP-C from centromeres after exposure to TRAIL. In preliminary experiments after transfection of INCENP, we observed a rapid degradation of wild-type INCENP that was not prevented by 50 μ M MG132 (data not shown). This loss of INCENP was due to a caspase-mediated cleavage, because exposure of control cells to z-VAD.fmk resulted in an accumulation of transfected INCENP (Figure 6A, lanes 1 and 3). Exposure to TRAIL also induced a loss of overexpressed wild-type INCENP as well as cleav-

age of endogenous CENP-C, both of which were prevented by z-VAD.fmk (Figure 6A, lanes 1, 2, and 4). Under these conditions, TRAIL induced the processing/loss of caspase-7 zymogen, which was also prevented by z-VAD.fmk (Figure 6A). In cells expressing high levels of noncleavable INCENP, TRAIL treatment resulted in similar levels of caspase-mediated cleavage of CENP-C and procaspase-7 (Figure 6A, lanes 5–8). However, the noncleavable INCENP was significantly more potent than wild-type INCENP at protecting cells from TRAIL-induced loss of CENP-C from centromeres as detected by confocal microscopy (Figure 6B). This suggests that INCENP cleavage may be required for the release of CENP-C cleavage products from the kinetochore (see schematic in Figure 6C), although it could also reflect differences in the efficiency of CENP-C cleavage at different stages of the cell cycle, because in the microscopy experiments loss of CENP-C was measured only in G₂/M cells (~15% of the total cell population), whereas the Western blot analysis measured CENP-C in the total population.

DISCUSSION

Experiments performed in yeast and vertebrate cells have revealed an important functional link between the chromosomal passenger proteins and kinetochores during mitosis. However neither kinetochores nor the passenger proteins

Figure 6. Transfection of noncleavable INCENP protects against the loss of centromeric CENP-C. (A) HeLa cells were transfected with wild-type and noncleavable D438A HA-tagged INCENP. The cells were then either untreated (UT) or exposed to $1 \mu\text{g ml}^{-1}$ TRAIL (T) for 2 h either alone or in the presence of $20 \mu\text{M}$ z-VAD.fmk (Z) and then subjected to Western blotting with an INCENP, CENP-C, or caspase-7 antibody. (B) HeLa cells transfected with either wild-type (WT) or the noncleavable INCENP mutant (D-A) were similarly exposed to TRAIL for the indicated times and labeled with antibodies against CENP-C and HA tag and scored for CENP-C loss ($n = 4$; 100–300 G2 cells were counted in each experiment). This treatment resulted in a significant difference in the median percentage of CENP-C loss in wild-type compared with noncleavable INCENP (D-A) ($p < 0.05$). (C) Schematic representation of the consequences of caspase-mediated cleavage of INCENP and CENP-C. Asterisk represents cleaved CENP-C or INCENP and mut represents the noncleavable mutant.

are known to have a function, either jointly or separately, during interphase. The present study reveals that, surprisingly, there is a functional link between kinetochores and the chromosomal passengers during interphase by showing that interfering with caspase-mediated cleavage of either can influence the localization of the other.

During mitosis, the passenger complex is essential for error correction at kinetochores: when passenger proteins are inactivated by mutation, depletion, or chemical inhibitors, cells undergo a striking increase in the number of syntelic and merotelic kinetochore attachment errors. In budding yeast, the kinetochore protein Dam1p is phosphorylated by the Aurora kinase Ipl1p, and this phosphorylation is important for the correction of attachment errors (Cheeseman *et al.*, 2002a). In vertebrates, microtubule binding by the kinetochore involves the Ndc80 complex, one of whose four components, Hec1/Ndc80, is phosphorylated by Aurora B (Cheeseman *et al.*, 2006; DeLuca *et al.*, 2006). Aurora B phos-

phorylation weakens the affinity of Hec1/Ndc80 for microtubules (Cheeseman *et al.*, 2006) and is essential for error correction during mitosis (DeLuca *et al.*, 2006). Action of Aurora B on MCAK or Inner Centromere Kin1 Stimulator (ICIS) may also have a role in error correction during mitosis (Ohi *et al.*, 2003; Andrews *et al.*, 2004; Lan *et al.*, 2004). Thus, the role of the chromosomal passenger complex in regulating kinetochore function in mitosis is beginning to be understood in some detail. In contrast, next to nothing is known about the role, if any, of centromeres or the passenger proteins during interphase.

Caspase-mediated Destruction of Key Centromeric Components Early in Apoptosis

At early times after TRAIL or TNF stimulation, active caspase-8 is detected in nuclear foci. In those cells, CENP-C was cleaved by caspases at the sequence DEAD↓L. INCENP was also cleaved and the chromosomal passenger complex was delocalized. Cleavage of CENP-A, CENP-B, Aurora B, Survivin, or Borealin/Dasra B was not detected. Although the caspase responsible for CENP-C cleavage *in vivo* could not be identified unequivocally, a variety of evidence suggested that caspase-7 was responsible and a knockdown of caspase-7 protected against the loss of CENP-C in TRAIL-treated MCF-7 cells. It is worth noting that after DNA damage, caspase-7 is the major caspase responsible for the cleavage of Claspin and the resulting inactivation of the Chk1 signaling pathway (Clarke *et al.*, 2005). However, most of our studies were carried out in MCF-7 cells, which are deficient in caspase-3. Therefore, we cannot exclude an additional role for caspase-3 in the cleavage of CENP-C and INCENP in other cell types where caspase-3 may be the major effector caspase.

Functional Consequences of CENP-C and INCENP Cleavage

The known interactions between chromosomal passengers and kinetochore proteins all occur during mitosis, whereas the cleavage of CENP-C and INCENP described here after TNF-R or TRAIL ligation occurs during interphase. Until very recently, centromeres had not been known to have any activities or functions during interphase. However, it has been recently discovered that the cleavage of centromeric components induced by Herpes simplex virus ICP0 (Everett *et al.*, 1999; Lomonte *et al.*, 2001) induces an interphase centromere damage response, which involves the functional reorganization of Cajal bodies and GEMs (Lomonte, personal communication). Although the consequences of this response are yet to be determined, such a reorganization within the nucleus could have functional consequences, including alterations in RNA processing and gene expression. Thus, it seems that centromeres and their associated chromosomal passenger complex may have a previously unrecognized role in the interphase nucleus, much as the condensin complex has recently been shown to also have a function in DNA repair (Heale *et al.*, 2006).

We have also considered other possible consequences of caspase cleavage of centromere components during interphase. Caspase-7-mediated cleavage of INCENP at Asp 438 produces two fragments of ~65 kDa. The C-terminal fragment contains the highly conserved IN-box (Adams *et al.*, 2001) that is responsible for binding and activation of Aurora B (Bishop and Schumacher, 2002; Honda *et al.*, 2003) but that lacks amino-terminal motifs required for targeting INCENP to centromeres during mitosis (Ainsztein *et al.*, 1998). Thus, caspase cleavage could lead to production of a deregulated kinase that might promote the cell death re-

sponse, as has been shown for caspase cleavage of a number of other protein kinases, including protein kinase C isoforms, mitogen-activated protein kinase kinase 1, p21-activated kinase 2, and Mst1 (Earnshaw *et al.*, 1999; Cheung *et al.*, 2003).

Although the present study has focused on events during interphase, it is also possible that the cleavage of CENP-C and INCENP described here is part of a mechanism to enforce the cell death decision by ensuring the failure of mitosis. For example, the destruction of CENP-C and CENP-A caused by Herpes simplex ICP0 leads to a dramatic disruption of mitotic events (Everett *et al.*, 1999; Lomonte *et al.*, 2001). In addition, BubR1 is cleaved by caspases during extended checkpoint arrest in HeLa cells, and this abrogates the checkpoint response, leading to an aberrant mitotic exit (Kim *et al.*, 2005). Mutation of the caspase cleavage sites in BubR1 seemed to reinforce the spindle checkpoint and also protected cells against apoptosis (Kim *et al.*, 2005). Interestingly, cleavage of INCENP and the resulting disruption of the chromosomal passenger complex would also be expected to interfere with the checkpoint response, because Survivin and Aurora B are required for stable checkpoint activation in response to the lack of spindle tension (Carvalho *et al.*, 2003; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Lens *et al.*, 2003).

Implications for Regulation of Kinetochores by the Chromosomal Passenger Complex

The N terminus of INCENP is sufficient for targeting of the protein to centromeres, possibly through interactions with Borealin and Survivin (Ainsztein *et al.*, 1998; Gassmann *et al.*, 2004; Vader *et al.*, 2006). However, the chromosomal receptor for the passenger complex remains unknown. Although CENP-A phosphorylation was implicated in the timely release of the INCENP and Aurora B from anaphase kinetochores (Zeitlin *et al.*, 2001a), the targeting and stability of kinetochores and chromosomal passenger proteins at centromeres were thought to be independent events. For example, RNAi depletion of CENP-A in *Caenorhabditis elegans* had no effect on INCENP localization to centromeres and vice versa (Oegema *et al.*, 2001). This was consistent with immunoelectron microscopy results, which revealed INCENP to be concentrated in the heterochromatin beneath the kinetochore (Cooke *et al.*, 1987), whereas CENP-A is located in the inner kinetochore plate (Cooke and Earnshaw, unpublished data).

The link between CENP-C and INCENP cleavage and localization discovered in the present study was therefore very unexpected. We have shown that both proteins are cleaved early in apoptosis and that the cleavage of CENP-C is independent of INCENP cleavage. Nonetheless, expression of noncleavable CENP-C rescued passenger targeting to the centromere and expression of noncleavable INCENP rescued CENP-C targeting to the kinetochore. Thus, CENP-C becomes the first structural protein shown to be required for localization of the chromosomal passenger complex to centromeres. Although expression of noncleavable INCENP did not block CENP-C cleavage in the bulk population, it is possible that it interferes with CENP-C cleavage at mitotic kinetochores. Alternatively, in the presence of noncleavable INCENP, the CENP-C cleavage fragments may persist at the kinetochore.

Why previous studies had failed to detect this dependence is not clear. The most obvious possibility is that additional components of the centromere are cleaved by caspases in our experiments and that this cleavage is required for loss of the passenger proteins from centromeres. If so, then it must be true that either the cleavage of these hypothetical proteins

or the loss of the passenger proteins requires cleavage of CENP-C. Alternatively, it is possible that low levels of residual kinetochore proteins remaining in RNAi studies might suffice for passenger targeting to centromeres and that CENP-C destruction by caspase-7 might simply be more efficient than RNAi. Finally, it is possible that the gradual depletion of kinetochore components that follows upon destruction of their mRNAs might allow cells sufficient time to adapt and up-regulate alternative pathways to influence chromosomal passenger localization, whereas the rapid cleavage that occurs during apoptosis does not.

In summary, after the induction of death receptor-mediated apoptosis, we have shown a very rapid appearance of active caspases within the nucleus that brings about the cleavage and loss of CENP-C and INCENP and the mislocalization of the other chromosomal passenger proteins. In the future, it will be important to determine whether this is part of the newly discovered interphase centromere damage response (Lomonte, personal communication), and if so, what the functional consequences of this response are for cell growth and survival.

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