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Letter to the Editor

Characterization of apoptosis-induced Mcm3 and Cdc6 cleavage reveals a proapoptotic effect for one Mcm3 fragment

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Dear Editor,

The initiation of DNA replication is a tightly controlled process, depending on the coordinated assembly and function of a specific set of proteins, the prereplicative complex (pre-RC) at chromosomal origin sequences. Integral components of this complex are Cdc6 and the six MCM proteins, Mcm2–7. MCM proteins may serve as replicative helicases. Once replication has started, reinitiation until the next round of the cell cycle is in part prevented by changes in the subcellular localization of Cdc6 and MCM proteins.[1,2] Limiting the availability of replication factors is thus an established pathway to prevent DNA replication.

Apoptosis is an important control mechanism to balance cell proliferation against cell death in development, differentiation and homeostasis in all multicellular organisms.[3] Its ATP-dependent execution prevents the leakage of potentially harmful intracellular contents as seen in necrosis. Halting of ATP-consuming processes like DNA replication could therefore further the efficiency of apoptosis.[4] Indeed, cleavage of replication proteins by caspases, the executioner proteases for apoptosis, has been reported.[5]

Here we show in detail the cleavage of the pre-RC proteins Mcm3 and Cdc6 by caspase-3 and caspase-7, including characterization of the cleavage sites. Localization as well as binding properties of the protein fragments generated in apoptotic cells are discussed, and a reinforcing proapoptotic mechanism for one of the Mcm3 fragments is described.

After the induction of apoptosis in the Burkitt's lymphoma cell line BL60-2, HeLa cells or the T cell line H9 by various stimuli, MCM proteins and Cdc6 were analyzed by immunoblotting. Proteolytic cleavage of Mcm3 and Cdc6 was observed in all cases (Figure 1a, upper and middle panel), while Mcm2 and Mcm4–7 remained unchanged (data not shown). The observed Mcm3 fragment has an apparent size of $\sim$90 kDa in agreement with previous reports.[7,8] The main cleavage product of Cdc6 had an apparent size of $\sim$45 kDa with a second fragment of $\sim$35 kDa appearing later in the apoptotic process (Figure 1a, middle panel). In response to each apoptotic stimulus, cleavage of replication initiation proteins was accompanied by activated caspase-3, the main executioner caspase in apoptotic cells (Figure 1a, lower panel). Further *in vitro* analysis using recombinant proteins identified caspase-3 and caspase-7 as the proteases capable to cleave Mcm3 and Cdc6 (see supplementary information). To identify the actual sites used for caspase cleavage, we performed site-directed mutagenesis of potential caspase recognition sites and assayed *in vitro* translated Cdc6 and Mcm3 protein fragments for proteolysis by recombinant caspase-3. For Mcm3, mutation of the aspartic acid corresponding to amino-acid 701 in the full-length protein to alanine resulted in the prevention of cleavage. A corresponding epitope tagged protein expressed in HeLa cells proved to be resistant to proteolysis (Figure 1b, upper panel). We therefore identified the caspase-3 site in Mcm3, used for cleavage in apoptotic cells, at $DAKD^{701}$. The caspase cleavage sites in Cdc6 were identified in a similar manner. We were able to locate caspase-3 cleavage sites in Cdc6 at $DEMD^{287}$ and $SEVD^{442}$ and, again, overexpressed mutant proteins were not cleaved in apoptotic HeLa cells (Figure 1b, lower panel). Next, we tried to express recombinant forms of the Mcm3 cleavage products in addition to the caspase-resistant mutant of the protein. However, we could not detect the small C-terminal Mcm3 fragment in HeLa cells or any other cell line tested. For the other constructs, coimmunoprecipitation analysis showed interaction with the endogenous Mcm4 protein, indicative for the incorporation of the truncated or mutant Mcm3 forms in MCM complexes (Figure 1c). In addition, all of the overexpressed Mcm3 related proteins analyzed were shown to localize to the nucleus in HeLa cells (see supplementary information). These overexpression experiments also revealed a strong proapoptotic effect for the large N-terminal Mcm3 cleavage product (Mcm3 1–700), as monitored by fluorescence activated cell sorter analysis (FACS) for caspase activity (Figure 1d and experimental details therein). In contrast, transfection of the full-length Mcm3 constructs (either wild type or caspase resistant) induced only a moderate increase of caspase activity in this highly sensitive assay when compared to mock-transfected cells. This finding indicates a reinforcing proapoptotic activity for the apoptotic, N-terminal Mcm3 fragment that might be involved in the sustaining of signaling cascades or even enhance the execution of apoptosis.

In analogy to our Mcm3 approach, we generated expression vectors coding for the N- and C-terminal Cdc6 fragments as well as the full-length protein and its caspase-resistant counterpart (see Figure 1e for an overview of Cdc6 versions). In immunofluorescence experiments, the intracellular localization of full-length Cdc6, its uncleavable version as well as of the N-terminal fragment was either nuclear (colocalization

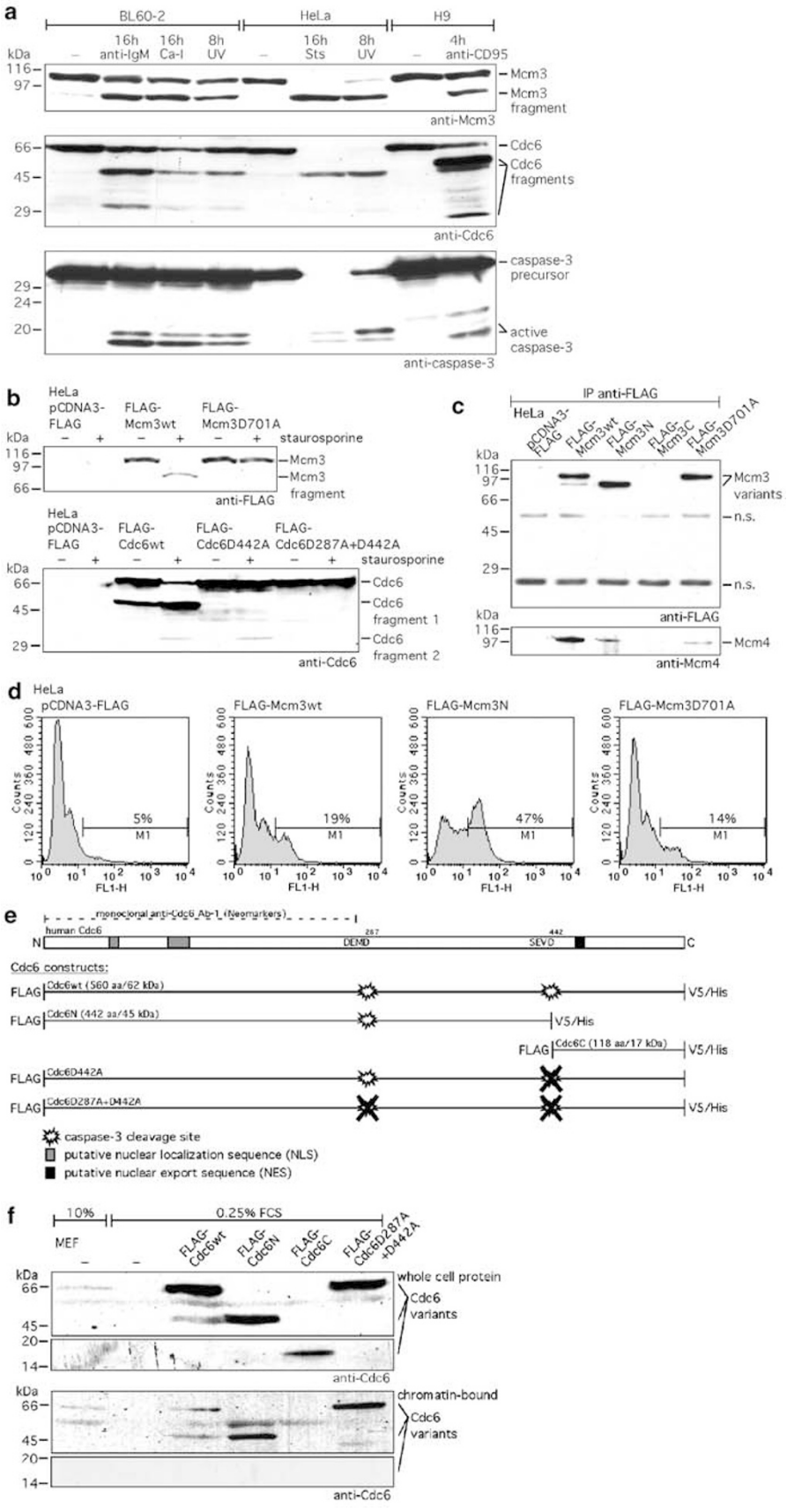
a
BL60-2
HeLa
H9
16h anti-IgM
16h Ca-I
8h UV
16h Sts
8h UV
4h anti-CD95
kDa
Mcm3
Mcm3 fragment
anti-Mcm3
Cdc6
Cdc6 fragments
anti-Cdc6
caspase-3 precursor
active caspase-3
anti-caspase-3
b
HeLa
pCDNA3-FLAG
FLAG-Mcm3wt
FLAG-Mcm3D701A
staurosporine
anti-FLAG
FLAG-Cdc6wt
FLAG-Cdc6D442A
FLAG-Cdc6D287A+D442A
fragment 1
fragment 2
c
IP anti-FLAG
FLAG-Mcm3N
FLAG-Mcm3C
Mcm3 variants
n.s.
anti-Mcm4
d
FLAG-Mcm3N
FLAG-Mcm3D701A
Counts
5%
19%
47%
14%
M1
FL1-H
e
monoclonal anti-Cdc6 Ab-1 (Neomarkers)
human Cdc6
DEMD
SEVD
Cdc6 constructs:
Cdc6wt (560 aa/62 kDa)
Cdc6N (442 aa/45 kDa)
Cdc6C (118 aa/17 kDa)
Cdc6D442A
Cdc6D287A+D442A
V5/His
caspase-3 cleavage site
putative nuclear localization sequence (NLS)
putative nuclear export sequence (NES)
f
10%
0.25% FCS
MEF
whole cell protein
chromatin-bound

Figure 1

with DAPI) or cytoplasmic. In contrast, the localization of the C-terminal fragment was exclusively cytoplasmic (see supplementary information), most likely as a result of the truncation of the nuclear localization signals located in the N-terminal part of Cdc6 (Figure 1e, see also Delmolino *et al*[9]). Interestingly, both the N- and C-terminal fragments generated upon induction of apoptosis in HeLa cells transfected with the wild-type Cdc6 expression construct localized exclusively to the cytoplasm (see supplementary information). To further characterize the apoptotic Cdc6 fragments and the cleavage-resistant Cdc6, we analyzed their capability to bind chromatin in comparison to wild-type Cdc6, when overexpressed in starved murine embryonal fibroblasts, which downregulate the endogenous Cdc6 protein.[10] All versions of the Cdc6 protein that were localized to the nucleus (see above) were able to bind to chromatin (Figure 1e).

While this work was in progress, an additional caspase-3 cleavage site in the Cdc6 protein was reported at LVFD^{99}.[11] We therefore tried to visualize the corresponding apoptotic Cdc6 fragment of $\sim$52 kDa described. The fragment was not detectable in apoptotic cell extracts with our anti-Cdc6 antibody, which binds in the N-terminal half of the protein. It was also not visible after generation of radiolabeled full-length Cdc6 in a coupled *in vitro* transcription/reticulocyte lysate translation system and subsequent cleavage of the protein with recombinant caspase-3, circumventing the use of antibodies. Only one large fragment of about 45 kDa was generated from wild-type Cdc6 (data not shown). In addition, we overexpressed in HeLa cells both wild-type Cdc6 and the caspase-resistant Cdc6 protein, each epitope tagged at both the N- and C-terminus. After induction of apoptosis, cell extracts were analyzed by immunoblots using the epitope-directed antibodies. Again, only the fragments observed before could be identified (see supplementary information). Using the experimental conditions in our study, this excludes apoptotic Cdc6 cleavage sites other than DEMD^{287} and SEVD^{442}.

Taken together, we were able to show cleavage of the pre-RC components Mcm3 and Cdc6 in different cell lines in response to various apoptotic stimuli. Mechanistically, such a process could ensure the inhibition of DNA replication in dying cells, thus preserving ATP for the apoptotic processes. The latter is likely to be important as ATP depletion was shown to switch apoptotic to necrotic cell death with its known side effects.[3] Furthermore, we were able to show a reinforcing proapoptotic function for the large apoptotic Mcm3 fragment. Therefore, the cleavage of Mcm3 might have a function in apoptosis *per se*, exceeding the inhibition of DNA replication.

Figure 1 (**a**) Mcm3 and Cdc6 are cleaved in response to various apoptotic stimuli. BL60-2 cells were cultured as described.[6] HeLa cells were cultured in Dulbecco's MEM with 10% FCS and 25 μg/ml gentamicin. H9 cells were cultured in RPMI-1640 with 10% FCS and gentamicin. Whole-cell protein extracts (SDS lysis) were prepared from BL60-2 cells, HeLa cells and H9 cells before and after induction of apoptosis with 1.3 μg/ml goat anti-human IgM F(ab)$_2$ antibody, 250 μM calcium ionophore A 23187, 120 mJ/cm^2 UV light, 1 μM staurosporine or 1 μg/ml anti-CD95 antibody followed by crosslinking F(ab)$_2$ goat anti-mouse IgG antibody, respectively. Cleavage of Mcm3, Cdc6 and caspase-3 was analyzed on immunoblots. (**b**) Overexpressed Mcm3 and Cdc6 with mutated caspase sites are not cleaved in apoptotic cells. FLAG-tagged Mcm3wt, Cdc6wt or the respective mutants with the identified caspase site(s) destroyed by site-directed mutagenesis were overexpressed in HeLa cells. Apoptosis was induced 18 h past transfection by addition of 1 μM staurosporine. After additional 6 h whole-cell protein was prepared and the extracts were analyzed by immunoblots. (**c**) Mcm3 variants overexpressed in HeLa cells bind to endogenous Mcm4. At 24 h past transfection HeLa cells were harvested, resuspended in CSK buffer (10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM ATP plus complete protease inhibitor cocktail), incubated on ice for 20 min and centrifuged at 800 $\times$ *g* for 5 min at 4°C. The supernatant, containing the soluble nonchromatin bound proteins, was incubated with anti-FLAG biotinylated M5 monoclonal antibody and subsequently with streptavidine-coated magnetic beads. Eluted fractions were analyzed on immunoblots for the overexpressed Mcm3 variants and coprecipitated endogenous Mcm4. (**d**) The N-terminal Mcm3 fragment exhibits a proapoptotic effect on HeLa cells. At 24 h past transfection HeLa cells were harvested, incubated in medium with CaspACE-FITC-VAD-fmk (Promega) for 30 min at 37°C to stain for activated caspases and analyzed by flow cytometry. (**e**) Cdc6 caspase-3 cleavage sites and expression constructs. Caspase-3 cleavage sites were identified in human Cdc6 protein at SEVD^{442} and DEMD^{287}. Cdc6 constructs used for expression in HeLa cells are depicted. (**f**) Chromatin binding of overexpressed Cdc6 variants in MEF cells. Murine embryonal fibroblasts (MEF) were cultured in Dulbecco's MEM with 10% FCS and gentamicin. FLAG-tagged Cdc6 variants were overexpressed in starved MEF cells (0.25% FCS), which downregulate endogenous Cdc6 protein. At 28 h past transfection cells were harvested and split in half. Whole-cell protein was prepared from one pellet. Chromatin-bound proteins were prepared by resuspending the second pellet in cold CSK buffer (see above) and centrifuging at 800 $\times$ *g* for 5 min at 4°C. The resulting pellet was boiled in SDS lysis buffer. Proteins of both preparations were analyzed and compared on immunoblots

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)