Letter to the Editor

Human spindle checkpoint kinase Bub1 is cleaved during apoptosis

Cell Death and Differentiation (2005) 12, 827–830. doi:10.1038/sj.cdd.4401620
Published online 8 April 2005

Dear Editor,

Apoptosis is a tightly regulated process of cell death that takes place in higher eukaryotes, and plays a key role in the development and homeostasis of an organism. Most of the morphological and biochemical changes exhibited by apoptotic cells are caused by the action of an evolutionarily conserved family of cysteine proteases that cleave their substrates after aspartic acid residues (caspases; reviewed by Earnshaw *et al.*¹). Effector caspases 3, 6 and 7 are the main executioners of the destruction of a cell by proteolytic degradation of a limited subset of intracellular proteins.²

Here, we investigated the possible connection between the spindle checkpoint machinery and apoptosis. The spindle checkpoint monitors the integrity of spindle–kinetochore attachments at prometaphase, ensuring that chromosomes are properly aligned at the mitotic spindle before proceeding into anaphase (reviewed by Gorbsky³). In the presence of a single unattached chromosome, or in response to lack of tension between spindle microtubules and kinetochores, a wait anaphase signal is induced. Central genes involved in the spindle checkpoint (Bub1, Bub3, Mad1, Mad2, Mad3/BubR1 and Mps1) are conserved in evolution and localize to kinetochores during mitosis (reviewed by Cleveland *et al.*⁴).

After induction of apoptosis in HL60 cells by treatment with the topoisomerase II inhibitor etoposide, the status of several spindle checkpoint proteins was analyzed by Western blotting. Proteolytic cleavage of Bub1, but not Bub3 or Mad1, was observed (Figure 1a). Full-length Bub1 protein was cleaved to an ~45-kDa fragment and an intermediate fragment of 90 kDa, only detected at early stages of apoptosis execution (Figure 1a, upper panel), indicating cleavage of Bub1 in the amino- and carboxy-terminal regions of the protein. Cleavage of Bub1 coincided with PARP cleavage (Figure 1a, second lower panel), with an increase in sub-G1 population (measured by flow cytometry) and with the onset of DNA laddering (data not shown). As a control, we show that Ku70, a component of the DNA-dependent protein kinase, remained intact (Figure 1a, lower panel). Proteolytic cleavage of Bub1 was also detected in HeLa cells treated with TNF- α and cycloheximide (see Supplementary Information). The fact that Bub1 cleavage occurs in different cell lines under different apoptotic conditions indicates that Bub1 cleavage is a universal process during apoptosis.

When HL60 cells were induced to undergo apoptosis in the presence of the broad-range caspase inhibitor ZVAD-fmk, Bub1 cleavage was abolished (Figure 1b, upper panel). In contrast, inhibitors of other classes of proteases had little or

no effect (data not shown). Bub1 was also cleaved in HL60 whole-cell extracts incubated *in vitro* with recombinant caspase 3, and the *in vitro* cleavage product had the same electrophoretic mobility as the band seen in etoposide-treated HL60 cells (Figure 1b, lower panel). Moreover, Bub1 protein was only partially cleaved in caspase 3-deficient MCF-7 cells treated with TNF- α and cycloheximide, in conditions of complete PARP cleavage (see Supplementary Information), suggesting that caspase 3 is the most important protease involved in Bub1 cleavage, although some other protease(s) may participate in this process. Indeed, recombinant caspases 3, 6 and 7 were able to cleave two different GST-Bub1 fusion fragments (GST-Bub1Nt, comprising amino acids 1–705; GST-Bub1Ct, amino acids 691–1085) (Figure 1c and data not shown). Our results, both *in vitro* and *in vivo* (see Figure 1a–c), led us to think that there are three caspase cleavage sites in Bub1 sequence, around positions 400, 490 and 700. We mutated all candidate aspartate residues to glycine in the GST-Bub1 fusion proteins and incubated these proteins with recombinant caspases, analyzing the results by Western blotting. We found that aspartates 395, 488 and 704 were targets for caspases (Figure 1c). When aspartate 395 was mutated to glycine, fragment 'x' disappeared, while fragment 'y' remained (Figure 1c, upper panel, lanes 5 and 6). GST-Bub1Nt construct carrying the double mutation in positions 395 and 488 was not cleaved by any caspase (lanes 8 and 9). Mutation of aspartate 702 partially abolished the cleavage of GST-Bub1Ct by caspase 3 (fragment 'z' in Figure 1c, middle panel, lane 5), while mutation of aspartate 704 resulted in a complete inhibition of this cleavage (lane 8). A summary of these results is shown in Figure 1c, lower panel. Notably, the recognition sequence around positions 395 and 704 diverges from the consensus recognition sequence for caspases 3, 6 and 7. Interestingly, cleavage by caspases releases an amino-terminal fragment containing the kinetochore localization and Bub3-binding domain, a central region with unknown function and a carboxy-terminal fragment containing the entire kinase domain. Furthermore, we also found, by immunoprecipitation in control and etoposide-treated HL60 extracts with antibodies raised against the two ends of the protein, that Bub1 amino- and carboxy-terminal apoptotic fragments do not interact with each other after cleavage (Figure 1d).

In order to study the physiological relevance of Bub1 cleavage, we first assayed the kinase activity of both the full-length protein and the apoptotic carboxy-terminal fragment

(amino acids 705–1085), using different kinase substrates (histone H1, histone H3 and PHAS-1; Figure 1e). We found that Bub1 carboxy-terminal fragment was able to phosphorylate

late histone H3, PHAS-1 and the light chain of the immunoprecipitating antibody to a higher extent than the full-length protein, but a higher kinase activity was observed

with the full-length protein when using histone H1 as a substrate. Our results suggest that release of Bub1 kinase domain by apoptotic cleavage does not produce a constitutively active kinase (as is the case of hPAK65/PAK2⁵) or a constitutively inactive kinase, as it happens with Akt/PKB.⁶ Instead, Bub1 kinase activity becomes deregulated after cleavage, and, therefore, the function of the protein inside the cell might also change.

To study the effects of Bub1 cleavage on spindle checkpoint, U2OS cells were transiently transfected with plasmids encoding for Bub1 full-length protein or the apoptotic amino- and carboxy-terminal fragments (amino acids 1–705 and 705–1085, respectively), and cotransfected with a GFP-spectrin-encoding plasmid. Transfected cells were either treated with a low dose of the microtubule-depolymerizing drug nocodazole or were left untreated, and the DNA contents of GFP-positive cells were measured by flow cytometry (Figure 1f). Cells transfected with an empty vector or full-length Bub1 accumulated in G2/M when treated with nocodazole. However, cells overexpressing Bub1 amino- or carboxy-terminal fragments were more resistant to nocodazole treatment, not accumulating in G2/M to the same extent, thus suggesting an over-ride of the checkpoint in these conditions. These effects could be due to a dominant-negative effect of the overexpressed fragments, and can mimic the total cleavage of Bub1 protein. Therefore, we suggest that Bub1 cleavage may cause inactivation of the spindle checkpoint during apoptosis.

Last, we studied the effect of Bub1 cleavage on apoptotic induction, by cotransfecting HEK-293T cells with a GFP-encoding plasmid together with amino-terminal, carboxy-terminal and full-length Bub1-expressing plasmids, and looking for apoptotic morphology of transfected cells. The three Bub1 constructs gave rise to a small apoptotic induction (4–7% in Bub1-expressing cells, compared to 1% in empty

vector-transfected cells; see Supplementary Information). These results suggest that deregulation of Bub1 function contributes itself to apoptosis to a certain extent. However, it is likely that cleavage of Bub1 is not essential for the direct induction of apoptosis, as is the case of other proteins that are degraded during apoptosis, such as ICAD or hPAK65/PAK2.^{5,7} Instead, considering all our results, we propose that apoptotic cleavage of Bub1 resembles that of ATM or DNA-PKcs (kinases involved in DNA damage checkpoint and DNA repair, respectively^{8,9}), as it causes inactivation of a genomic integrity-maintaining mechanism that is no longer necessary when apoptosis is triggered. We also speculate that Bub1 cleavage does not cause massive cell death by itself, but only in combination with other cellular modifications. For example, Rad21, one of the subunits of the cohesin complex, which keeps sister chromatids together before anaphase, is also cleaved during apoptosis.¹⁰ Under these conditions, inactivation of the spindle checkpoint could increase apoptotic cell death. In fact, when we overexpressed Bub1 amino- and carboxy-terminal fragments in the presence of nocodazole, we observed an increase of apoptosis measured as an increase of sub-G1 population (Figure 1f). It has also been proposed that mechanisms that preserve the intracellular levels of ATP could favor apoptosis, since ATP depletion has been shown to switch cell death from apoptosis to necrosis.¹¹ Inactivation of an ATP-consuming process such as the spindle checkpoint could contribute to apoptosis through this mechanism.

Acknowledgements

We thank B Vogelstein (The Johns Hopkins Oncology Center, Baltimore) for the human BUB1 cDNA. Caspases 3, 6 and 7 cDNAs were a kind gift from GS Salvesen at the Burnham Institute (La Jolla, CA). We are grateful

Figure 1 (a) Cleavage of human Bub1 protein during apoptosis. HL60 cells, cultured in RPMI 1640 medium supplemented with 15% FCS, 100 U/ml penicillin and 10 U/ml streptomycin, were incubated with 68 μ M etoposide and collected at the indicated times. Whole-cell extracts (SDS lysis) were prepared and analyzed by Western blotting with the following antibodies: anti-Bub1-Nt,¹² anti-Bub3, anti-Mad1 (raised in rabbit against a recombinant His-tagged fragment of Mad1 protein spanning amino acids 1–290), anti-PARP and anti-Ku70. (b) Cleavage of Bub1 protein is caspase-dependent. HL60 cells were left untreated, treated with 68 μ M etoposide for 3 h or treated with etoposide in the presence of 40 μ M ZVAD-fmk. After treatment, whole-cell extracts were prepared and analyzed by Western blotting with the anti-Bub1-Nt antibody (upper panel). HL60 cells were either left untreated or treated with 68 μ M etoposide for 2 or 3 h. Whole cell extracts were prepared from untreated HL60 cells, and incubated *in vitro* with recombinant His-tagged caspase 3 for the indicated times, in the presence or absence of 40 μ M ZVAD-fmk (lower panel). (c) Identification of Bub1 caspase cleavage sites. Recombinant GST-tagged Bub1Nt wt (amino acids 1 to 705; upper panel), GST-Bub1Ct wt (amino acids 691 to 1085; middle panel) or the indicated mutants were incubated *in vitro* with recombinant caspases 3, 6 and 7. The reaction products were analyzed by Western blotting using an anti-GST antibody. Arrows show intact GST-Bub1 fragments and the faster migrating bands obtained after cleavage by caspases (x, y and z). Schematic diagram showing Bub1 cleavage sites (lower panel). CD1, N-terminal homology domain; KLD, kinetochore localization and Bub3-binding domain; CD2, serine-threonine kinase domain. (d) Bub1 amino- and carboxy-terminal apoptotic fragments do not interact after cleavage. After a 4 h treatment with 68 μ M etoposide, HL60 cells were lysed in buffer III (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 mM sodium orthovanadate, 50 μ g/ml ethidium bromide plus protease inhibitor cocktail). The extracts obtained were then subjected to immunoprecipitation, using anti-Bub1-Nt, anti-Bub1-Ct or control IgG antibodies, and protein A-Sepharose CL4B (Amersham Biosciences). HL60 whole-cell extracts (Input) and immunoprecipitated proteins were analyzed by Western blotting with the anti-Bub1-Nt antibody. The heavy chain of the antibody used for immunoprecipitation is shown. (e) Modulation of Bub1 kinase activity after cleavage by caspases. HEK-293T cells (grown in Dulbecco's MEM supplemented with 10% FCS, 100 U/ml penicillin and 10 U/ml streptomycin) were transiently transfected with myc-tagged full-length Bub1 (BUB1), carboxy-terminal Bub1 fragment (amino acids 705–1085; BUB1Ct) or control vector pcDNA3.1/myc-His (V). After 24 h, cells were lysed as in (d) and subjected to immunoprecipitation with an anti-myc antibody and anti-mouse IgG-conjugated magnetic beads (Dynal). Kinase activity of equal amounts of immunoprecipitated proteins (quantified by Western blot) was analyzed using 5 μ Ci of [γ -³²P]ATP (Amersham Biosciences) and 500 ng of histone H1, histone H3 or PHAS-1 as substrates. The reaction products were subjected to electrophoresis and analyzed by autoradiography. Arrows show the bands representing each of the substrates. Asterisks mark the band corresponding to the light chain of the antibody used for immunoprecipitation. (f) U2OS cells (grown in Dulbecco's MEM supplemented with 10% FCS, 100 U/ml penicillin and 10 U/ml streptomycin) were transiently transfected with full-length Bub1 (BUB1), amino-terminal Bub1 fragment (amino acids 1–395; BUB1Nt), carboxy-terminal Bub1 fragment (amino acids 705–1085; BUB1Ct) or an empty vector (Vector), and cotransfected with a spectrin-GFP expression plasmid. At 48 h after transfection, cells were treated for 16 h with 15 ng/ml nocodazole. Cells were then collected and DNA contents of the GFP-positive cells were determined by flow cytometry, using a Coulter Epics XL bivariate flow cytometer (Beckman Coulter)

to SP Jackson for the Ku70 antibody and V Smits (Erasmus MC, Rotterdam) for technical advice. This work was supported by grants from FIS (Fondo de Investigaciones Sanitarias) (01/1624), from the EU (HPRN-CT-2002-00240) and from the Spanish Ministry of Science and Technology (SAF2004-07856) to RF. DP was supported by a predoctoral fellowship from the Spanish Ministry of Education and Culture. RF is currently supported by a FIS (Fondo Investigaciones Sanitarias) contract.

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