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Role of factors downstream of caspases in nuclear disassembly during apoptotic execution

Kumiko Samejima, Pascal Villa and William C. Earnshaw*

Institute of Cell and Molecular Biology, University of Edinburgh, Kings' Buildings, Mayfield Road, Edinburgh EH9 3JR, UK

We used cytoplasmic extracts from chicken DU249 cells at various stages along the apoptotic pathway to analyse the events of apoptotic execution. So-called S/M extracts from morphologically normal 'committed-stage' cells induce apoptotic morphology and DNA cleavage in substrate nuclei. These apoptotic changes appear to require the function of multiple caspases (cysteine aspartases, a specialized class of proteases) acting in parallel. Extracts from 'execution-stage' apoptotic cells induce apoptotic events in added nuclei in a caspase-independent manner. Biochemical fractionation of these extracts reveals that a column fraction enriched in endogenous active caspases is unable to induce DNA fragmentation or chromatin condensation in substrate nuclei, whereas a caspase-depleted fraction induces both changes. 'Execution-stage' extracts contain an ICAD/DFF45-inhibitable nuclease resembling CAD, plus another activity that is required for the apoptotic chromatin condensation. 'Committed-stage' S/M extracts lack these downstream activities. These observations reveal that caspases act in an executive fashion, serving to activate downstream factors that disassemble the nucleus rather than disassembling it themselves. They also suggest that activation of the downstream factors (rather than the caspases) is the critical event that occurs at the transition from the latent to the execution phase of apoptosis.

Keywords: apoptosis; caspases; CAD; ICAD; DFF

1. INTRODUCTION

Apoptosis is a major form of physiological cell death characterized by morphological and biochemical changes including cellular disassembly and the activation of proteases and DNases. The apoptotic pathway is activated by proteases, of which the cysteine aspartases (caspases) play the best-characterized role (Cohen 1997; Nicholson & Thornberry 1997; Villa *et al.* 1997; Cryns & Yuan 1998). Genetic analyses show clearly that the sole *Caenorhabditis elegans* caspase, CED-3, is essential for all apoptotic death in that organism (Yuan & Horvitz 1990; Kuida *et al.* 1996; Woo *et al.* 1998). Fourteen members of the caspase superfamily have been found in mammals (Cohen 1997; Villa *et al.* 1997; Nicholson & Thornberry 1997; Cryns & Yuan 1998; Earnshaw *et al.* 1999). Out of these, caspases 1, 4, 5, 11 and 14 appear to be involved primarily in the inflammatory response, whereas caspases 2, 3, 6, 7, 8, 9 and 10 appear to function during cell death.

Despite intensive study in many laboratories, the role of this enzyme family in the death pathway remains unclear. One recent study has suggested that cells can activate caspases without undergoing apoptosis (Boise & Thompson 1997). Conversely, even though caspase inhibitors usually rescue cells from apoptosis (reviewed by Villa *et al.* 1997), cell death can occur in response to proapoptotic stimuli in the presence of these inhibitors (Xiang *et al.* 1996; McCarthy *et al.* 1997; Lavoie *et al.* 1998). Recent genetic studies have shown that caspases 3 and 9 are particularly important components of the apoptotic pathway in the developing mouse brain (Kuida *et al.* 1996, 1998; Woo *et al.* 1998; Hakem *et al.* 1998). Nonetheless, careful examination has revealed that cells dying in the presence of caspase inhibitors display membrane blebbing and cell-surface alterations, but no changes in nuclear morphology (McCarthy *et al.* 1997). This observation suggests that certain cytoplasmic hallmarks of apoptosis may be triggered by enzymes other than caspases, but that nuclear events require caspase activity. Consistent with this view, cells from caspase-3-null mice have been reported to display plasma membrane changes and cleavage of the nuclear protein poly(ADP-ribose) polymerase when undergoing apoptosis, but not the chromatin condensation and DNA cleavage that are characteristic of apoptosis (Woo *et al.* 1998). A similar result has been reported for the caspase-3-null cell line MCF-7 (Jänicke *et al.* 1998*a*,*b*).

Taken together, the preceding observations suggest that certain caspases play a central role in apoptotic events, especially those occurring in the nucleus. However, it is not known whether caspases function in an executive role to initiate the apoptotic pathway and leave the work of actually disassembling the cell to other downstream factors, or whether they are workhorses whose cleavage of key substrates drives cellular disassembly.

At present approximately 40 substrates for caspases during apoptosis have been identified (Cohen 1997; Villa *et al.* 1997; Nicholson & Thornberry 1997; Cryns & Yuan 1998; Porter *et al.* 1997). Many of these are involved in cell signalling pathways (Earnshaw *et al.* 1999); for most, the role of their cleavage in apoptotic execution is unknown.

*Author for correspondence (bill.earnshaw@ed.ac.uk).

One key caspase substrate is the recently discovered protein ICAD/DFF (Enari *et al.* 1998; Liu *et al.* 1997). ICAD/DFF is an obligate binding partner of the caspase-activated DNase CAD/CPAN (Enari *et al.* 1998; Halenbeck *et al.* 1998; Mukae *et al.* 1998). During translation, ICAD acts as a chaperone, whose presence is essential for the proper folding of CAD. The nuclease is catalytically inactive in the complex with ICAD/DFF (Enari *et al.* 1998; Sakahira *et al.* 1998). This complex has been proposed to be retained in the cytoplasm of healthy cells (Enari *et al.* 1998). Cleavage of ICAD by a caspase such as caspase 3 is thought to unmask a cryptic nuclear localization signal on CAD and permit the newly activated enzyme to enter the nucleus and degrade the genomic DNA (Enari *et al.* 1998; Sakahira *et al.* 1998; Liu *et al.* 1997).

Current understanding of caspase function has been facilitated by the development of cell-free systems for the study of apoptosis (Lazebnik *et al.* 1993; Solary *et al.* 1993; Newmeyer *et al.* 1994; Enari *et al.* 1995; Martin *et al.* 1995; Schlegel *et al.* 1995; Liu *et al.* 1996). One such system developed in our laboratory uses extracts from chicken DU249 hepatoma cells that become committed to apoptosis following perturbation of the cell cycle (Wood & Earnshaw 1990; Lazebnik *et al.* 1993). Highly concentrated cytosolic extracts prepared from morphologically normal DU249 cells ('S/M' extracts) reproduce all of the biochemical features of apoptosis in substrate nuclei (Lazebnik *et al.* 1993), including genome digestion (Wyllie *et al.* 1980; Lazebnik *et al.* 1993), cleavage of a subset of nuclear proteins, including PARP and lamins (Kaufmann 1989; Ucker *et al.* 1992; Lazebnik *et al.* 1994, 1995), chromatin condensation and nuclear fragmentation into apoptotic bodies (Lazebnik *et al.* 1993). This entire programme of apoptotic events is inhibited *in vitro* by caspase inhibitors (Lazebnik *et al.* 1994) or millimolar concentrations of Zn^{2+} (Lazebnik *et al.* 1993), just as it is in intact cells. Although nuclei are used as the substrate in these studies, the extracts themselves are derived from the cytoplasm of the DU249 cells (Lazebnik *et al.* 1993), thus supporting the view that cytoplasmic factors and events have an essential role in the apoptotic pathway (Jacobson *et al.* 1994; Schulze-Osthoff *et al.* 1994; Nakajima *et al.* 1995; Martin *et al.* 1996; Kroemer 1997). It should be noted that these extracts, which are centrifuged at 150 000 *g* for 2 h during preparation, do not contain mitochondria or other membranous organelles.

In the studies described here we prepared extracts from chicken DU249 cells at various stages of the apoptotic pathway to further evaluate caspase involvement in nuclear disassembly. Extracts prepared from morphologically normal cells in the latent phase (S/M extracts) and those prepared from frankly apoptotic cells ('execution phase extracts') induced similar apoptotic events in exogenous nuclei but exhibited fundamental biochemical differences. In particular, apoptotic events in the S/M extracts were abolished by caspase inhibitors, as previously reported (Lazebnik *et al.* 1994), whereas the same events in execution-phase extracts were unaffected by inhibition of the caspases. Further studies have revealed that execution-phase extracts contain at least two caspase-activated factors required for nuclear disassembly, one of which appears to be a CAD-like nuclease. These experiments have recently been described in greater detail by Samejima *et al.* (1998).

To study the regulation of CAD by ICAD in more detail we generated a GFP–ICAD fusion protein, which, surprisingly, had a nuclear and not a cytoplasmic localization in intact cells. We extended these observations by performing cell-fractionation experiments that confirmed the nuclear localization of ICAD. These experiments have recently been described in greater detail (Samejima & Earnshaw 1998).

Together, the experiments presented here support the view that caspases act in an executive role in nuclear apoptosis by activating downstream factors that then disassemble nuclei. They also suggest that activation of those downstream factors (rather than the caspases) accompanies the transition between the latent and execution phases of apoptosis. Finally, our studies prompt us to reassess previous models, which stated that the major function of ICAD was cytoplasmic.

2. METHODS

All methods used in this paper have been described in detail previously (Samejima *et al.* 1998; Samejima & Earnshaw 1998).

3. RESULTS AND DISCUSSION

(a) *Extracts from different stages of apoptosis display differences in dependence on ongoing caspase activity*

Apoptosis is a two-phase process. Upon receipt of a proapoptotic signal, cells enter a 'latent' phase in which they follow a pathway, as yet poorly understood, that culminates in death. Cells in the latent phase appear morphologically normal. For the purpose of these studies, we have arbitrarily subdivided the latent phase into two stages. Upon receipt of a proapoptotic signal, we suggest that cells enter a 'condemned' stage. In these cells, the death programme has been initiated, but the cells can be rescued by various survival factors. Once cells pass a point of no return, they are in the 'committed' stage and can no longer be rescued. Eventually, committed cells undergo an abrupt transition into apoptotic 'execution', a relatively brief period lasting under an hour during which cellular disassembly and death occur. It is not known where along this pathway caspases are activated or whether, and if so at what point, the caspases activate other downstream factors that act during disassembly of the cell (Samejima *et al.* 1998).

To analyse biochemical events occurring during these different stages of apoptosis, we harvested chicken DU249 cells at various times after subjecting cultures to a cell-synchronization protocol shown previously to induce an apoptotic response in these cells (Lazebnik *et al.* 1993). Briefly, cells were presynchronized in S phase with aphidicolin for 12 h, released from this block for 6 h, and then subsequently harvested 3 h after the addition of nocodazole to the culture. Cells harvested at this point were then used to prepare concentrated cell-free extracts by means of the freeze–thaw–grind approach described in earlier publications (Wood & Earnshaw 1990; Lazebnik *et al.* 1993). C/D ('condemned' phase) extracts were prepared from the morphologically normal interphase

cells that remained adherent at the end of the procedure. Previous studies have indicated that many of these cells ultimately undergo apoptosis if left in culture (Lazebnik *et al.* 1993). S/M ('committed' phase) extracts (Lazebnik *et al.* 1993) were prepared from morphologically normal mitotic cells obtained by selective detachment after 3 h in nocodazole. Because these cells are destined to rapidly undergo apoptosis if left in culture, and because they contain high concentrations of active caspases (Samejima *et al.* 1998), we postulate that S/M extracts reproduce events from the committed stage of apoptosis. E/X ('execution' phase) extracts were prepared from cells that became apoptotic during the synchrony procedure and were obtained by shake-off before the addition of nocodazole.

C/D extracts were unable to induce internucleosomal DNA fragmentation and apoptotic morphological changes in exogenous nuclei (Samejima *et al.* 1998). They have thus been used as controls throughout the experiments described below. In contrast, S/M and E/X extracts induced hallmark biochemical and morphological changes of apoptosis in added nuclei (figure 1).

Despite the fact that S/M and E/X extracts have similar caspase activity, as detected with a fluorogenic tetrapeptide substrate (Samejima *et al.* 1998), and produce effects on added nuclei (figure 1), the two extracts displayed strikingly different properties after inhibition of their endogenous caspase activity. Pretreatment of S/M extracts with DEVD-fmk before addition of nuclei not only inhibited caspase activity (Samejima *et al.* 1998) but also completely abolished the ability of the extracts to induce internucleosomal DNA fragmentation (figure 1*a*, lane 4) and apoptotic morphological changes (figure 1*b*, panel 4). In striking contrast, pretreatment with DEVD-fmk inhibited the caspase activity but not the apoptosis-inducing activity of E/X extracts (figure 1*a*, lane 6; figure 1*b*, panel 6).

These experiments reveal a fundamental biochemical difference between extracts made from committed-phase cells and those made from cells in apoptotic execution. The fact that extracts from execution-phase cells are able to strongly induce an apoptotic morphology in added nuclei even after the inhibition of caspase activity strongly suggests that nuclear disassembly is triggered not by the caspases themselves, but instead by other activities that operate downstream of the caspases. These downstream activities appear to be fully induced in execution-phase cells. In contrast, although committed-phase cells contain active caspases, they apparently do not contain appreciable levels of the downstream activities. Thus, extracts from these cells require continuous caspase activity to produce an apoptotic phenotype in added nuclei.

This requirement for caspase activity could have two explanations. First, it might be that in committed-phase S/M extracts it is the caspases themselves that disassemble the nuclei. Alternatively, it could be that in these extracts the downstream activities become activated *in vitro* as a consequence of caspase activity. Preincubation experiments described in detail elsewhere (Samejima *et al.* 1998) support this latter conclusion, at least for the apoptotic nuclease CAD. Thus, it appears that committed-phase S/M extracts contain largely inactive CAD, which is progressively activated during incubations *in vitro*, provided that caspase activity is maintained.

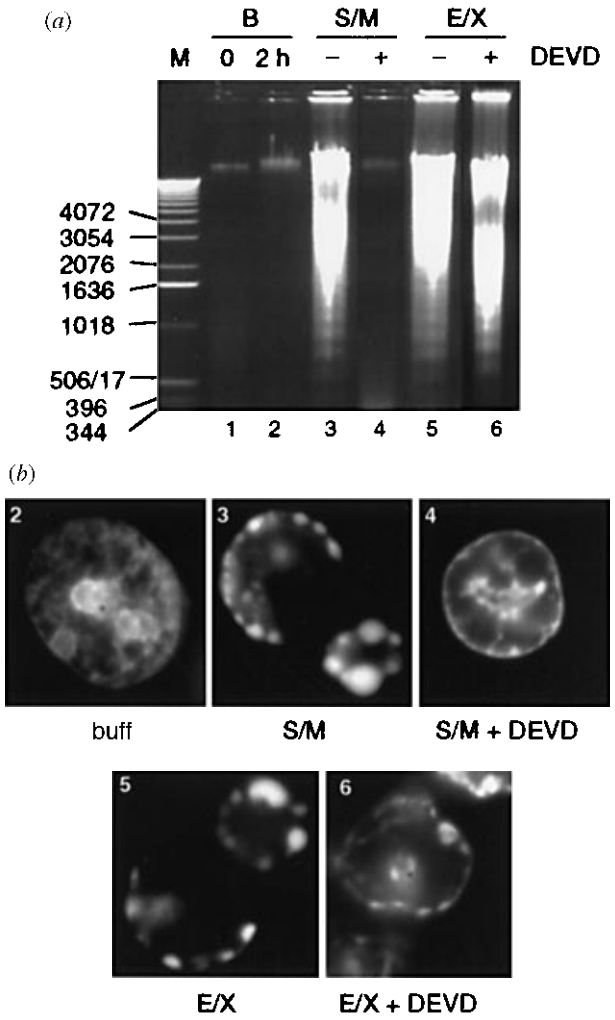

Figure 1. Caspase inhibitors block apoptosis in S/M but not E/X extracts. (*a*) Inhibition of caspases blocks nuclease activity in S/M ('committed phase') extracts but not E/X ('execution phase') extracts. B, buffer control. (*b*) Inhibition of caspases abolishes morphological apoptosis in vitro in S/M extracts but not E/X extracts.

A second line of experimentation also supports the notion that nuclei are disassembled by activities downstream of caspases, and not by the caspases themselves. In other studies, we have fractionated large-scale committed-phase S/M extracts, producing a fraction that appeared to contain the full complement of endogenous caspases, and a second fraction in which only traces of caspase activity could be detected (Samejima *et al.* 1998). The caspase-rich fraction proved to be completely unable to induce either DNA degradation or morphological changes in added nuclei, whereas the caspase-poor fraction was extremely active in the induction of apoptosis *in vitro*, even after all caspase activity had been abolished by incubation with DEVD-fmk.

The studies reported here, together with previous work from our laboratory, have shown that nuclear disassembly is not accomplished by the caspases themselves, but instead by other activities that function downstream of caspases. The caspases are active in committed-phase cells, but appear to be sequestered from the downstream activities, as the latter are only activated *in vitro* after lysis

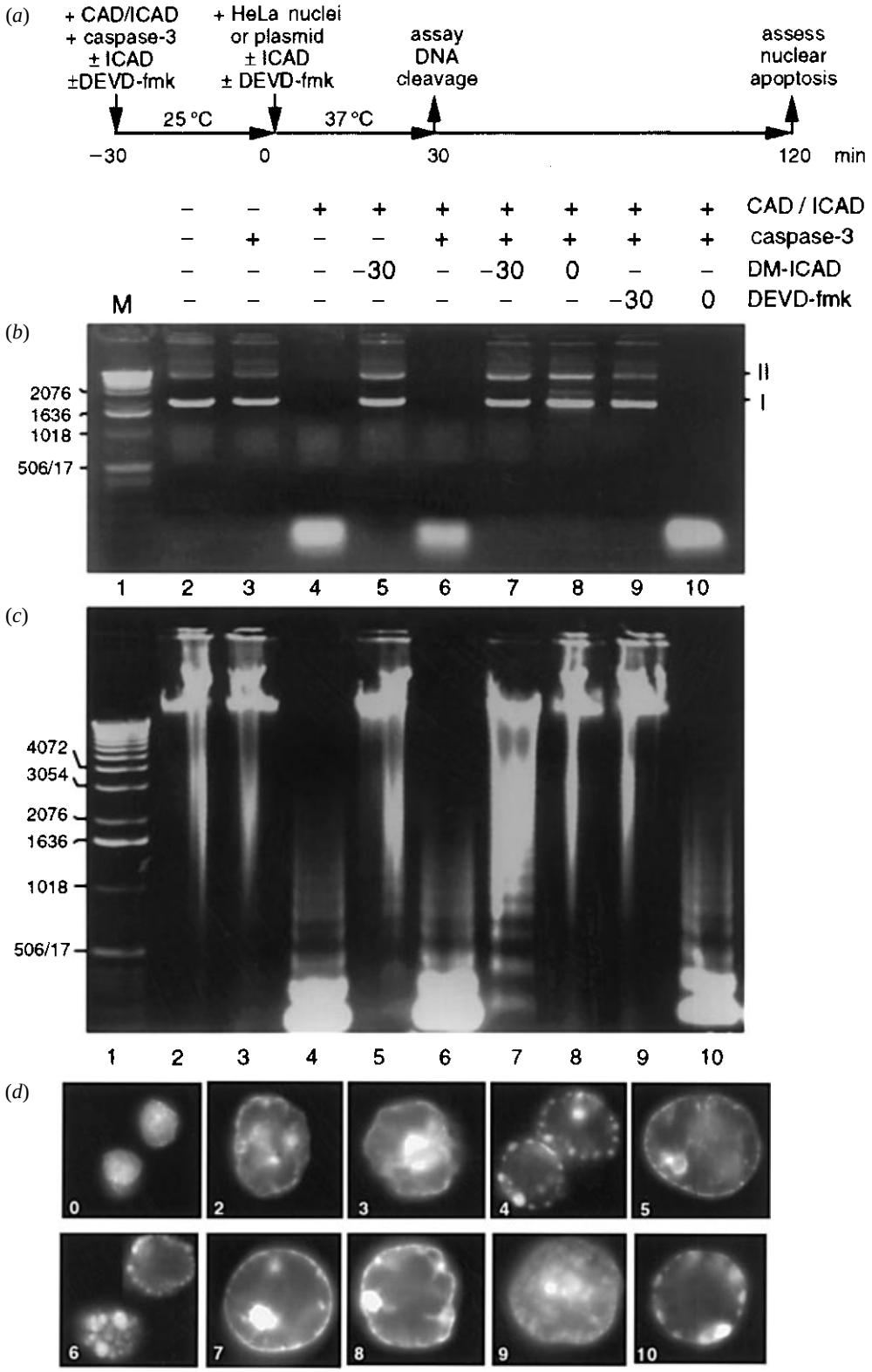

Figure 2. Bacterially expressed CAD is capable of inducing apoptotic morphology in added nuclei in the absence of apoptotic extract. (*a*) Diagram of the experimental protocol. CAD is activated during a preincubation with purified caspase 3 at 25 °C, then substrates are added and incubated for various times with the active enzyme at 37 °C. (*b*,*c*) Effects of various incubations on CAD nuclease activity with either a purified pBluescript plasmid substrate (*b*) or added nuclei (*c*). The morphology of the nuclei in these same incubations is shown in (*d*), where the panel numbers refer to lanes in (*b*) and (*c*). Buffer and caspase 3 do not possess nuclease activity or induce apoptotic morphology in added nuclei (lanes 2 and 3). Preincubation of CAD alone induces nuclease activity and apoptotic morphology (lane 4), presumably owing to the presence of a contaminating protease. ICAD is cleaved during this preincubation (data not shown). This spontaneous activation of CAD is suppressed if non-cleavable ICAD is present from the start of the preincubation (lane 5). Preincubation of CAD with caspase 3 induces nuclease activity and apoptotic morphology (lane 6). This activity is suppressed if non-cleavable ICAD is present either from the start of the preincubation, or if it is added at the end of the preincubation together with the substrate nuclei (lanes 7 and 8). Addition of DEVD-fmk to the

of the cell. In contrast, lysates from execution-phase cells contain active downstream factors that no longer require caspase activity for full function. Thus the transition from the committed to the condemned phase of apoptosis in cells appears to be accompanied by the activation of the downstream factors that ultimately drive the disassembly of the nucleus.

One key question raised by these observations is the identity of those downstream factors. As shown below, one such important factor is the apoptotic nuclease CAD.

(b) *CAD on its own is able to induce apoptotic morphology in added nuclei*

Because the results presented in figure 1 suggested that the programme of nuclear disassembly is initiated by caspase activity but does not require the ongoing participation of caspases for its successful execution, we next turned our attention to caspase-activated downstream factors that might participate in nuclear disassembly. One of these activities is the recently identified caspase-activated deoxyribonuclease CAD/CPAN, identified in both murine and human cells undergoing apoptosis (Enari *et al.* 1998; Halenbeck *et al.* 1998; Mukae *et al.* 1998).

One of the important initial observations in early studies of apoptosis was the finding that the DNA is degraded to a nucleosomal ladder during the apoptotic death of thymocytes (Wyllie 1980). This was the first evidence that the biochemical changes during apoptosis were specific and not simply reflective of general autolysis of the cell. Both S/M and E/X extracts contain an endogenous DNase activity that degrades plasmid DNA and causes the DNA of added nuclei to be degraded to a characteristic nucleosomal ladder (Lazebnik *et al.* 1993). This nuclease apparently corresponds to CAD: addition of purified murine ICAD completely abolishes the activity of the endogenous nuclease in extracts (Samejima *et al.* 1998; Enari *et al.* 1998; Sakahira *et al.* 1998). This result highlights the strong degree of evolutionary conservation in apoptotic systems, because the murine ICAD is able to specifically inhibit chicken CAD.

The experiment shown in figure 2 confirms that CAD is a caspase-activated factor that can induce nuclear morphological changes during apoptotic execution. To perform this experiment, we developed a bicistronic vector for expression of the ICAD–CAD complex in *Escherichia coli*. Use of such a vector ensured that the cloned CAD was translated in the presence of an excess of ICAD. The ICAD–CAD complex was purified by nickel chelate chromatography and then tested for activity against plasmid and nuclei.

Results obtained with a plasmid substrate are shown in figure 2*b* (see the experimental protocol shown in 2*a*). The *E. coli*-expressed CAD became activated after a preincubation of 30 min at 25 °C in either the absence (figure 2*b*, lane 4) or the presence (lane 6) of purified recombinant caspase 3. *E. coli* lysates apparently contain an activity that is capable of inactivating ICAD. If a double mutant caspase-uncleavable ICAD, prepared as described by Sakahira *et al.* (1998), was added at the start of the preincubation, this blocked CAD activation in both the absence (lane 5) and presence (lane 7) of caspase 3. Double mutant ICAD also blocked CAD activity if added at the end of the preincubation (lane 8). These results were expected, because ICAD is capable of binding to CAD and inactivating it. Addition of DEVD-fmk at the start of the preincubation also blocked CAD activation (lane 9). However, addition of DEVD-fmk at the end of the preincubation (after cleavage of ICAD) had no effect on the CAD activity (lane 10), as expected, because ICAD was already cleaved at this point.

The plasmid assay thus confirmed that we were able to express CAD in *E. coli* under conditions that allowed the subsequent activation of the enzyme by caspase 3. We therefore used this preparation of partly purified CAD to examine the effects of CAD activity on added nuclei. As expected, bacterially expressed CAD was able to induce an oligonucleosomal ladder in added HeLa nuclei (figure 2*c*). This reaction behaved very similarly to the plasmid DNA assay, with one exception. Addition of double mutant caspase-uncleavable ICAD at the start of the preincubation only partly blocked CAD activation in both the absence (lane 5) and presence (lane 7) of caspase 3. Some factor or factors present in the nuclear preparations is apparently able to partly inactivate even the double mutant ICAD and release small amounts of active CAD.

Given the results of the DNA cleavage analysis, the key experiment was to ask what effect active CAD had on the morphology of HeLa nuclei. To do this, nuclei were removed from the reaction mixture befrore the DNA extraction used to prepare the samples for the gel shown in figure 2*c*. These nuclei were then stained with DAPI and examined under a fluorescence microscope. This experiment yielded a striking result. Partly purified CAD expressed in bacteria is able to strongly induce an apoptotic morphology in added HeLa nuclei (figure 2*d*). The numbered panels in figure 2*d* correspond to the gel lanes in figure 2*b*,*c*. Thus in samples where a strong oligonucleosomal ladder was observed (lanes 4 and 6) a strong induction of apoptotic morphology was also observed (panels 4 and 6). Interestingly, although the addition of DEVD at time $t=0$ had no effect on the production of the oligonucleosomal ladder (lane 10), it did slightly impair the morphological apoptosis (panel 10), although examples of fully apoptotic nuclei could also be seen in this sample. When nuclei exposed to CAD were examined by electron microscopy, the hypercondensed chromatin domains could be seen to bear a striking resemblance to chromatin domains produced during bona fide apoptosis (Samejima *et al.* 1998).

Figure 2. (*Cont.*) preincubation blocks CAD activation, but has no effect on nuclease activity or induction of apoptotic morphology if added once the preincubation is complete (lanes 9 and 10). The induction of apoptotic morphology by CAD in the presence of DEVD-fmk was slightly, but reproducibly, reduced (panel 10). These results demonstrate that CAD can induce nuclear apoptosis in the absence of caspase 3 activity.

Because caspase 3 was used to activate the bacterially expressed CAD, it was important to rule out the possibility that this caspase contributed to either the production of the oligonucleosomal ladder or the morphological changes seen in added nuclei. However, control experiments revealed that caspase 3 alone was unable to induce either DNA cleavage or apoptotic morphology in added nuclei (figure 2*b,c*, lane 3; figure 2*d*, panel 3).

This experiment, and others reported elsewhere (Samejima *et al.* 1998), indicate that CAD is one factor that operates downstream of caspases to produce biochemical and morphological changes in added nuclei. We were initially surprised that this nuclease was able to produce such dramatic morphological changes in nuclei: previous studies had shown that micrococcal nuclease, although able to induce some clumping of the chromatin, was unable to produce the collapse against the nuclear periphery and condensation into discrete spherical domains that are characteristic of apoptosis (Arends *et al.* 1990). The mechanism by which CAD selectively causes the condensing chromatin to collapse against the nuclear periphery is presently unknown.

One possible interpretation of these results was that CAD might be the only factor activated downstream of caspases that is required for nuclear disassembly. However, other experiments indicate that this is not so. We have shown elsewhere that addition of ICAD to E/X extracts has no effect on the ability of those extracts to induce morphological apoptosis in added nuclei, even under conditions where the cleavage of the nuclear DNA is completely abolished (Samejima *et al.* 1998). Thus, a second activity must operate in parallel with CAD to promote nuclear disassembly. At present the identity of this activity is unknown.

(c) *ICAD/DFF is nuclear in growing cells*

Cell fractionation experiments that led to the purification of ICAD/DFF used cytoplasmic fractions as a primary source of material (Liu *et al.* 1997; Enari *et al.* 1998). This ultimately led to the attractive model that ICAD/DFF is a cytoplasmic factor that, when cleaved upon caspase activation, releases a previously inactive cytoplasmic nuclease that subsequently enters the nucleus and degrades the cellular DNA (Enari *et al.* 1998). We decided to test this model by examining the location of ICAD in greater detail.

To examine the localization of ICAD in cells, we constructed a fusion protein between ICAD and the *Aequoria victoria* green fluorescent protein (GFP). This construct was used for both transient transfections and the production of stable cell lines. Both types of experiment yielded an unexpected result: ICAD/DFF is a nuclear protein (Samejima & Earnshaw 1998). In the example shown in figure 3*a*, the GFP–ICAD fusion protein was detected solely in the nucleus of healthy human HeLa cells after transient transfection. Similar results were obtained with pig and chicken cells (Samejima & Earnshaw 1998). GFP on its own does not target to the nucleus (data not shown; see also Rizzuto *et al.* 1995). Within the nucleus, the GFP–ICAD fusion protein showed a relatively homogeneous distribution, but was excluded from nucleoli. A similar result has recently been obtained by using transient transfection of epitope-tagged ICAD in human cells (Liu *et al.* 1998).

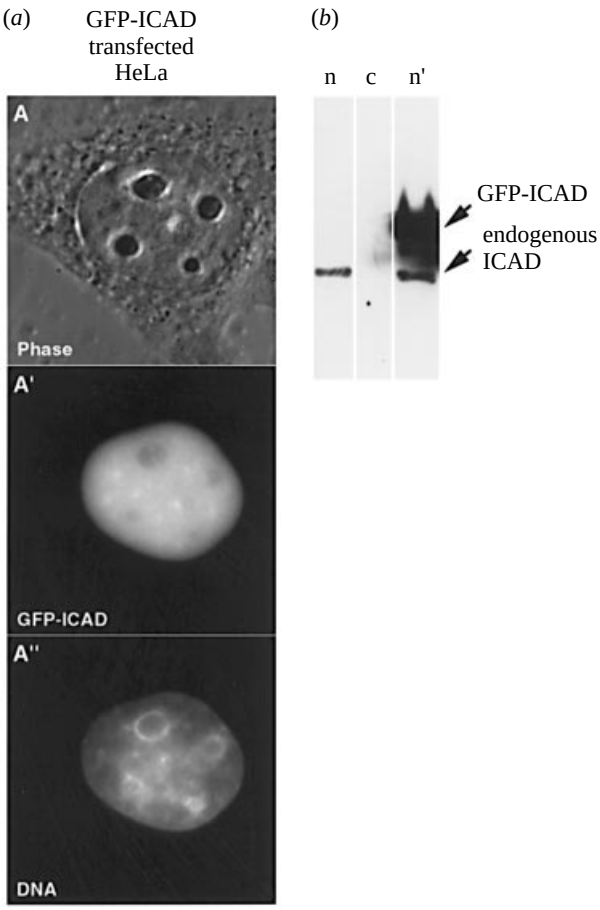

Figure 3. (*a*) GFP–ICAD is a nuclear protein. *Aequoria victoria* green fluorescent protein (GFP) was fused in frame to murine ICAD and expressed by transient transfection in human (HeLa) cells. The expressed protein was observed solely in cell nuclei. A, phase contrast; A′, GFP fluorescence; A″, DNA stained with DAPI. (*b*) Subcellular fractionation confirms that ICAD is a nuclear protein. Anti-ICAD recognizes endogenous human ICAD in a crude nuclear fraction prepared by centrifugation of whole cell extract at 2400 *g* for 10 min (lane n). ICAD is not detected in clarified cytosol (85 000 *g*, 1 h) (lane c). Anti-ICAD recognizes GFP–ICAD in a similar fractionation of transfected cells (lane n′).

Although this surprising nuclear localization of the GFP–ICAD fusion protein was extremely reproducible, it was nevertheless important to demonstrate that these results reflected the distribution of the endogenous ICAD/DFF. We therefore prepared two polyclonal antibodies that detect human, mouse and chicken ICAD in immunoblots (Samejima & Earnshaw 1998). The specificity of one of these ICAD antisera is demonstrated in figure 3*b*. The serum recognized a single polypeptide of *ca.* 45 kDa in nuclei isolated from HeLa cells (lane n). When nuclei were isolated from HeLa cells transfected with GFP–ICAD, the antibody recognized both the endogenous human ICAD/DFF and the 75 kDa GFP–ICAD fusion protein (lane n′). The antiserum did not recognize any polypeptides in a clarified cytosolic fraction prepared by sedimenting a postnuclear supernatant at 85 000 *g* for 1 h (lane c).

This localization of endogenous ICAD in nuclei was confirmed in other experiments where HeLa cells were lysed and subjected to a single centrifugation at 2400 *g* for 10 min (Samejima & Earnshaw 1998). That protocol fractionated cells into a crude nuclear pellet and total cytosol. The efficacy of the fractionation was confirmed by immunoblotting with antibodies to DNA topoisomerase I, a nuclear protein, and β-tubulin, a cytoplasmic protein. In several experiments the endogenous ICAD was found to be predominantly nuclear, with only a trace amount of the antigen being detected in crude cytosol. Given the presence of low levels of topoisomerase I in the cytoplasm in these experiments, we postulated that some or all of the cytoplasmic ICAD was likely to derive from nuclear breakage. This would be consistent with the absence of detectable ICAD in clarified cytosol (figure 3*a*).

These experiments strongly suggest that it is unlikely that ICAD functions solely in the cytoplasm, but they do not rule out the possibility that a small subfraction of the ICAD may function in the cytoplasm as a chaperone for CAD, as originally suggested (Enari *et al.* 1998). At present, we are in the midst of an extensive two-hybrid screen to look for other binding partners of ICAD in the cell nucleus. The results of these experiments may reveal whether the large nuclear pool of ICAD has an important function distinct from its ability to associate with CAD.

4. CONCLUSIONS

When we began these experiments, we believed, as many still do, that the role of caspases is to act directly on key cellular structures so as to bring about the dramatic structural reorganization of the cell that defines cell death by apoptosis. Earlier studies from our group had identified the first caspase substrate in apoptosis, PARP (Lazebnik *et al.* 1994) and had gone on to identify the lamins as key caspase substrates (Lazebnik *et al.* 1995; Takahashi *et al.* 1996*a*,*b*). However, the results of the present experiments have begun to change this view of the role of caspases. In experiments discussed here, we have demonstrated that endogenous activities present in cells undergoing apoptotic execution are fully competent to drive the nuclear disassembly characteristic of apoptosis in the absence of ongoing caspase activity. These 'downstream' activities, one of which is the nuclease CAD/CPAN, apparently require caspases for activation, but once switched on they function on their own to disassemble the nucleus. One key focus of future research in this area will be to identify the other activities that cooperate with CAD in nuclear disassembly.

In other experiments we have demonstrated not only that caspases are not required for nuclear disassembly, but also that a highly concentrated cocktail of the endogenous caspases from apoptotic cells is on its own unable to drive the apoptotic disassembly of nuclei (Samejima *et al.* 1998). So what is the primary role of caspases in apoptosis? We believe that the weight of current evidence supports the notion that caspases function in an executive role in apoptosis, first turning off a number of critical pathways that promote cell survival, and then turning on downstream activities that disassemble the cell (Samejima *et al.* 1998; Earnshaw *et al.* 1999). Examination of the broad spectrum of caspase substrates reveals that relatively few are likely to be directly involved in the disassembly of the cell. Instead, the caspases appear to function at crucial points to influence the decision of cells to commit to death.

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Discussion

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). I don't understand why these extracts are apoptotic in the first place, because, if you took these cells out of nocodazole, wouldn't they settle down and resume growth?

W. C. Earnshaw. No, these cells are committed to apoptosis after the aphidicolin treatment. They have one last fling before dying however. That is, they complete the cell cycle and die in the next cell cycle. This appears to be specific for this particular cell line.

J. Raff (*Wellcome—CRC Institute, Cambridge, UK*). Is it possible that caspases have a more direct role in the cytosolic events?

W. C. Earnshaw. It's clear that they are cleaving a number of cytoskeletal components. There are a couple of ways of looking at this. Caspases could be helping to 'take the cell apart'. On the other hand, many of the components of survival pathways are assembled on these structures. So, maybe they are actually acting to inactivate the survival pathways. Probably they are doing both. About 30 of the 40 or so known caspase substrates can be argued as falling into these regulatory pathways.

K. A. Nasmyth (*Research Institute of Molecular Pathology, Vienna, Austria*). Your hypothesis might predict that there will be another group of proteases activated downstream of the caspases.

W. C. Earnshaw. Yes, there are a number of proteases which are activated downstream, these include cathepsins and calpain for example. The caspases have drawn attention because they appear to be only active during apoptosis and can be shown by knockout experiments to be essential for apoptosis in certain systems.