Cleavage of Rabaptin-5 blocks endosome fusion during apoptosis

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Cells undergoing apoptosis exhibit striking changes in membrane organization, including plasma membrane blebbing and invagination, vacuolation and fragmentation of organelles, and alterations in the surface expression of receptors. The underlying mechanisms for these changes are unknown, though alterations in vesicular fusion are likely to play a role. Using a cell-free system based on *Xenopus laevis* egg extracts we have found that endosome fusion is blocked during apoptosis. Inhibition of fusion is prevented by Bcl-2 or Bcl-x_L, two negative regulators of apoptosis, or by specific inhibitors of members of the caspase family of apoptotic proteases. Selective cleavage of Rabaptin-5, an essential and rate-limiting component of endosome fusion, is responsible for the loss of fusion activity. Cleavage of Rabaptin-5 also occurs in cellular models for apoptosis. These results suggest that inactivation of Rabaptin-5 and inhibition of vesicle transport lead to fragmentation of endosomes and inhibition of the endocytic pathway during the execution phase of apoptosis. We propose that parallel changes to other membrane transport pathways would give rise to general membrane fragmentation in apoptotic cells. These changes are likely to play an important role in the generation of apoptotic bodies and their recognition by phagocytosing cells.

Keywords: apoptosis/Bcl-2/caspase/fusion/Rabaptin-5

Introduction

Apoptosis, or programmed cell death, is a conserved process which performs a number of functions in multicellular organisms, including maintenance of correct cell number, removal from tissues of damaged cells, and the operation of differentiation pathways (Chinnaiyan and Dixit, 1996; Raff, 1996). Cells undergoing apoptosis exhibit a characteristic series of changes, including DNA cleavage, nuclear breakdown, cell membrane blebbing and fragmentation of cytoplasmic organelles (Kerr *et al.*, 1972; Wyllie, 1980). Ultimately, these events lead to the fragmentation of the cell, generating apoptotic bodies that are swiftly engulfed by neighbouring cells within tissues,

preventing an inflammatory response. It is clear from the conservation of the process that execution of the apoptotic pathway requires a specific series of alterations to cellular function and changes in cell morphology that are important for recognition and removal of apoptotic cells.

The caspase family of cysteine proteases related to the *Caenorhabditis elegans* Ced-3 cell death gene product are known to play a crucial role in the apoptotic pathway (Thornberry *et al.*, 1992; Yuan *et al.*, 1993). These proteases cleave a number of proteins thought to play key roles in cellular processes that are altered during apoptosis. They have a highly selective substrate specificity defined by an essential aspartate residue at the site of cleavage (Thornberry *et al.*, 1992). Differential recognition by particular family members is determined by residues immediately N-terminal to the site of cleavage. Highly potent inhibitors that mimic these cleavage sites have been used to block the execution phase of apoptosis both in cells and cell-free systems (Martin *et al.*, 1995b; Nicholson *et al.*, 1995; Cosulich *et al.*, 1996).

The initiation of the apoptotic pathway needs to be tightly regulated. Activation of caspases occurs when they are proteolytically processed from inactive precursors. Interestingly, cleavage occurs at aspartate residues by similar activities, leading to the hypothesis that they are activated in a proteolytic cascade (Chinnaiyan and Dixit, 1996). Caspase activation can be triggered by a number of cellular stresses or by ligation of certain cell surface receptors, such as Fas/Apo-1 and TNF-R1 (Enari *et al.*, 1995; Boldin *et al.*, 1996; Muzio *et al.*, 1996). Recently, significant progress has been made towards understanding the regulation of caspase activation by these receptors. Initiating caspases are activated when assembled in a multi-component complex with the cytoplasmic domains of the receptors (Boldin *et al.*, 1996; Muzio *et al.*, 1996). The activation of caspases is also controlled by a family of proteins related to the Bcl-2 proto-oncogene product (Thornberry *et al.*, 1992; Chinnaiyan *et al.*, 1996; Cosulich *et al.*, 1996), which is able to protect cells from undergoing apoptosis (Nunez and Clarke, 1994; Reed, 1994). Other proteins within this family include Bcl-x_L, which also plays a negative regulatory role in the apoptotic pathway (Nunez and Clarke, 1994; Reed, 1994). In contrast, Bak and Bax are positive regulators of apoptosis (Oltavi *et al.*, 1993; Kiefer *et al.*, 1995). Bcl-2 family members associate with intracellular membranes, particularly mitochondrial membranes, and some have a structure related to pore-forming proteins (Muchmore *et al.*, 1996). However, their molecular mechanism of action is unknown at present.

Substrates for caspases that have been identified to date include enzymes involved in DNA damage recognition (PARP, DNA-PKcs, pRb) (Lazebnik *et al.*, 1994; Janicke *et al.*, 1996; Song *et al.*, 1996), structural proteins of the nuclear lamina (lamins) (Takahashi *et al.*, 1996), and

proteins involved in cytoskeletal organization (fodrin, Gas-2, Rho-GDI) (Brancolini *et al.*, 1995; Martin *et al.*, 1995b; Na *et al.*, 1996). However, the putative role played by the cleavage of many of these substrates in nuclear and cytoskeletal changes during apoptosis remains to be demonstrated. Furthermore, the protease substrates involved in other morphological changes, including membrane reorganization, are unknown. Since the maintenance of membrane organization is dynamic, changes in membrane fusion activity are likely to underlie these alterations.

We have previously characterized a cell-free system that reconstitutes endosomal membrane fusion (Woodman and Warren, 1988). In separate studies, we (Cosulich *et al.*, 1996) and others (Newmeyer *et al.*, 1994) have recently shown that a similar cell-free system of *Xenopus* egg extracts faithfully reproduces many apoptotic events, including activation of caspases, DNA fragmentation and characteristic changes in nuclear morphology. In this report, we show that endosomal fusion activity in these extracts declines as apoptotic proteases are activated. The decline in fusion activity is prevented by inhibitors of caspases, as well as Bcl-2 and Bcl-x_L, which block caspase activation in this system (Cosulich *et al.*, 1996). We have identified Rabaptin-5, an essential and rate-limiting component of fusion (Stenmark *et al.*, 1995), as a novel substrate for caspases. Cleavage of Rabaptin-5 blocks its function, accounting for the decline in membrane fusion. We therefore demonstrate how cleavage of a specific caspase substrate during apoptosis affects a defined cellular process.

Results

Endosome fusion declines during apoptosis in *Xenopus* egg extracts

Previous studies have shown that extracts from *Xenopus laevis* eggs support fusion between mammalian endosomes efficiently (Tuomikoski *et al.*, 1989). Upon incubation, similar extracts undergo biochemical changes indistinguishable from those observed in apoptotic cells (Newmeyer *et al.*, 1994; Cosulich *et al.*, 1996; Le Romancer *et al.*, 1996), including activation of caspases (Cosulich *et al.*, 1996). We therefore investigated whether there are changes in the ability of egg extracts to support endosome fusion during apoptosis. We found that incubation of extracts reduced significantly their ability to support endosome fusion (Figure 1A and B), measured by formation of immunocomplexes when endosomes containing labelled transferrin are incubated with endosomes containing anti-transferrin antibody (Woodman and Warren, 1988). Although the rate of decline of vesicle fusion varied somewhat between extract preparations, for any given extract the decline in fusion activity was always coincident with internucleosomal DNA fragmentation in added HeLa nuclei (Figure 1C and D). Notably, both events occurred after a similar lag period. Furthermore, the decline in fusion activity followed activation of caspases measured by cleavage of the fluorogenic substrate z-DEVD-AFC (Cosulich *et al.*, 1996 and data not shown). Therefore, it appeared likely that the effect on fusion activity reflected apoptosis-specific changes in the extracts.

In order to confirm this, we tested whether Bcl-2 or Bcl-x_L, two negative regulators of apoptosis (Nunez and

Fig. 1. Apoptosis in a cell-free system leads to inhibition of endosomal vesicle fusion. (A) *Xenopus* egg extract was incubated at 23°C and samples assayed after the indicated times for its ability to support endosomal vesicle fusion (□). The effects of Bcl-2 (50 µg/ml) on the decline in fusion activity were also assessed (■). Results are means of duplicate determinations. (B) A different extract was incubated as in (A), but with (■) or without (□) Bcl-x_L (10 µg/ml). (C and D) Samples from the same *Xenopus* egg extract as used in (A) or (B) respectively were treated as indicated, and then combined with HeLa nuclei and assayed for nucleosomal DNA fragmentation.

Clarke, 1994) that prevent apoptotic chromosome condensation and DNA fragmentation in *Xenopus* egg extracts (Newmeyer *et al.*, 1994; Cosulich *et al.*, 1996), allowed the extracts to maintain their fusion-supporting activity. Addition of Bcl-2 (Figure 1A) or Bcl-x_L (Figure 1B) to the extracts before incubation prevented the decline in fusion-supporting activity. Both Bcl-2 and Bcl-x_L prevented DNA fragmentation in parallel experiments (Figure 1C and D).

Specific caspase inhibitors prevent the decline in fusion-supporting activity

We have shown that apoptotic changes in nuclei added to *Xenopus* egg extracts can be completely blocked by peptide derivatives which are potent inhibitors of caspases (Thornberry *et al.*, 1992; Nicholson *et al.*, 1995; Cosulich *et al.*, 1996). The tetrapeptide aldehyde Ac-DEVD-CHO, an inhibitor of human caspase-3 (CPP32) and other members of the Ced-3 subfamily of caspases, completely prevented inhibition of membrane fusion when added to extracts at concentrations above 200 nM (Figure 2A). This is close to the concentration of Ac-DEVD-CHO required to prevent inactivation of DNA-dependent protein kinase (DNA-PK) (Le Romancer *et al.*, 1996) and cleavage of poly(ADP ribose) polymerase (PARP; S.Cosulich and P.Clarke, unpublished observations) in similar extracts. Interestingly, this differed from the concentration of Ac-

Fig. 2. Specific caspase inhibitors block decline in membrane fusion activity. (A) *Xenopus* egg extract was incubated for 5 h at 23°C with Ac-DEVD-CHO as indicated. Samples were assayed for their ability to support endosomal membrane fusion. The maximal activity is the same as that of an extract protected by addition of Bcl-2 at $t = 0$ (data not shown). (B) The modified tetrapeptide ZVAD-CH₂F was added to *Xenopus* egg extracts at the concentrations indicated and incubation continued for 5 h at 23°C. Samples were assayed for their ability to support endosomal vesicle fusion. (C) As (B), but with differing concentrations of Ac-YVAD-CHO.

DEVD-CHO required to prevent internucleosomal DNA fragmentation (10 nM; Cosulich *et al.*, 1996). ZVAD-CH₂F, another inhibitor of caspases (Fearnhead *et al.*, 1995), blocked both inhibition of membrane fusion and DNA fragmentation at a concentration of between 1 and 10 μM (Figure 2B and data not shown). In contrast, much higher concentrations (100–200 μM) of Ac-YVAD-CHO, a selective inhibitor of the interleukin-1β converting enzyme (ICE/caspase-1) subfamily of caspases (Thornberry *et al.*, 1992), were required to protect membrane fusion (Figure 2C). The rate of decline was unaffected by the presence of general protease inhibitors such as PMSF, E64, antipain, pepstatin A and chymostatin (data not shown). These results are fully consistent with a requirement for the activity of an apoptotic protease belonging to the caspase-

3/*C.elegans* Ced-3 subfamily of caspases for inhibition of membrane fusion.

Rabaptin-5 is a caspase substrate

Since we had shown that the decline in endosome fusion during apoptosis required caspase activity, we investigated whether a number of effectors of fusion might be cleaved by caspases. A downstream effector of Rab5, Rabaptin-5, was recently identified as an essential and rate-limiting component of fusion (Stenmark *et al.*, 1995). We found that *Xenopus* Rabaptin-5 was cleaved in egg extracts from a polypeptide with apparent molecular mass of 115 kDa to produce a fragment of ~50 kDa detected by an antibody raised against a C-terminal fragment of human Rabaptin-5 (Figure 3A). Cleavage was coincident with loss of endosome fusion activity (Figure 1A) and was completely prevented by Ac-DEVD-CHO added at the start of the incubation. The concentration of Ac-DEVD-CHO required to inhibit Rabaptin-5 cleavage (Figure 3B) was very similar to that which prevented the decline in fusion activity (Figure 2A). In contrast to Rabaptin-5, other cytosolic effectors of endosome fusion, including Rab5 (Gorvel *et al.*, 1991), Rab GDI (Ullrich *et al.*, 1994), NSF (Diaz *et al.*, 1989), and α-SNAP (Rodriguez *et al.*, 1994), were stable throughout the incubation (Figure 3C). Furthermore, there were no differences in overall protein stability apparent from Coomassie-stained gels (Figure 3D), indicating that generalized proteolysis was not occurring. It seemed likely, therefore, that inhibition of fusion was caused, at least in part, by selective cleavage of Rabaptin-5.

Further evidence for a link between Rabaptin-5 cleavage and inhibition of endosome fusion was obtained by addition to extracts of purified caspase-3 at sufficient concentration to accelerate the activation of endogenous *Xenopus* caspases. Figure 4A demonstrates that acceleration of Rabaptin-5 cleavage by 1–2 h occurred upon addition of caspase-3. Similarly, reduction of endosome fusion activity was observed 1–2 h earlier in the presence of caspase-3 (Figure 4B).

Cleavage of Rabaptin-5 causes loss of endosome fusion activity

We tested further the role of Rabaptin-5 cleavage in the loss of endosome fusion activity by reconstitution experiments. For Ac-DEVD-CHO to protect against loss of fusion activity, the protease inhibitor had to be added prior to incubation of extracts (which produced 'non-apoptotic' extracts). If the inhibitor was added after incubation (producing 'apoptotic' extracts), but before addition of membranes, fusion activity remained low. However, activity in apoptotic extracts could be restored towards initial levels by addition of non-apoptotic extracts. In contrast, further addition of apoptotic extracts did not increase fusion activity significantly (Figure 5A). Thus, inhibition of fusion can be accounted for by inactivation of factor(s) supplied by the extracts rather than components of the endosome membranes. To confirm that apoptotic extracts did not exert a 'dominant negative' effect on fusion activity caused by the presence of inhibitory factor(s), we combined different ratios of apoptotic and non-apoptotic extracts. The extent of fusion was inversely proportional to the amount of apoptotic extract in each sample (Figure 5B).

Fig. 3. Rabaptin-5 is cleaved in apoptotic extracts. (A) *Xenopus* egg extract was incubated for the indicated time with or without 2 μ M Ac-DEVD-CHO, then assayed for Rabaptin-5 immunoreactivity. (B) *Xenopus* egg extract was incubated for 5 h at 23°C with Ac-DEVD-CHO as indicated. Samples were analysed for Rabaptin-5 protein. (C) *Xenopus* egg extracts were incubated for 5 h at 23°C (lane 1) or 4°C (lane 2), then analysed by Western blotting for the indicated proteins. (D) *Xenopus* egg extracts were incubated for 5 h at 23°C (lane 1) or 4°C (lane 2), then analysed by SDS-PAGE followed by Coomassie blue staining.

Having established that non-apoptotic extracts could restore endosome fusion activity, we tested whether this was dependent on the presence of Rabaptin-5. Indeed, non-apoptotic extracts depleted of Rabaptin-5 were unable to restore activity (Figure 6A, left panel). Addition of recombinant Rabaptin-5 to these depleted extracts led to significant restoration of activity (Figure 6A, right panel).

While recombinant Rabaptin-5 alone provided only partial restorative activity to apoptotic extracts (Figure 6A, right), cytosolic fractions enriched in Rabaptin-5 and containing the Rabaptin-5-associated factor p60 (Horiuchi *et al.*, submitted) were fully able to restore activity (Figure 6B). Similar fractions did not alter significantly the activity of control extracts under these conditions. Restoration was dependent on Rabaptin-5; prior depletion of fractions with anti-Rabaptin-5 antibodies, but not control antibodies, removed the restorative activity (Figure 6C). One explanation for the restorative activity of this fraction, but not recombinant Rabaptin-5, is that p60 might also be cleaved during apoptosis. However, Western blot analysis using an antibody raised to a p60 peptide showed that a protein of 60 kDa, presumed to be *Xenopus* p60, was not cleaved in apoptotic extracts (Figure 6D). The inability of recombinant Rabaptin-5 to restore activity could be explained by the cleavage of additional, unidentified factor(s). Alternatively, recombinant Rabaptin-5 may simply have reduced activity in these extracts. In summary, these data demonstrate that Rabaptin-5 cleavage, at least in part, underlies the loss of endosome fusion activity.

Rabaptin-5 is cleaved in human cells during apoptosis

Since Rabaptin-5 has not been identified previously as a substrate for caspases during apoptosis, we wished to confirm that it is also cleaved during apoptosis in human cells. CEM-C7 cells treated with dexamethasone enter apoptosis between 24 and 56 h after administration of the drug, exhibiting an apoptotic phenotype including chromatin condensation and membrane ruffling (Norman and Thompson, 1977). In these cells, Rabaptin-5 immunoreactivity was lost between 24 and 48 h after addition of dexamethasone (Figure 7, upper panel). In contrast to *Xenopus* egg extracts, a cleavage product was not readily identified, indicating that further degradation of at least the C-terminal portion of the protein had occurred. Loss of Rabaptin-5 was selective, since levels of tubulin were

maintained throughout the course of the experiment (Figure 7, lower panel).

Cleavage of Rabaptin-5 was not confined to one cellular model for apoptosis. HL-60 cells treated with the topoisomerase inhibitor etoposide underwent apoptosis over several hours, and Rabaptin-5 cleavage was observed over a similar period. Approximately 80–90% of Rabaptin-5 was lost from cells within 4 h of addition of etoposide (Figure 8). Addition of Ac-ZVAD.CH₂F prevented this cleavage from occurring. Again, the unchanged levels of tubulin indicated that loss of Rabaptin-5 was selective. Similarly, only a slight reduction in levels of transferrin receptor were observed.

The more synchronous entry into apoptosis of etoposide-treated HL-60 cells allowed Rabaptin-5 cleavage to be correlated with other cellular changes. As shown in Figure 9, cells undergoing apoptosis exhibited characteristic changes in membrane morphology, including pronounced vacuolation of organelles within the cytoplasm (compare Figure 9A and B). Fragmentation of other membranes was clearly observed. Most notable is the loss of the nuclear envelope, which is entirely absent from condensed chromatin in apoptotic cells (compare Figure 9C and D). Golgi complexes, clearly distinguishable in control cells (Figure 9C) appeared to be absent from apoptotic cells. Some membrane structures were preserved in apoptotic cells; mitochondrial membranes were still distinguishable and some membrane remnants, presumably endoplasmic reticulum (ER), could be seen in the peripheral cytoplasm.

Although we have not specifically examined the morphology of endosomes in apoptotic cells, biochemical evidence suggests that the endocytic pathway is altered. A marked reduction in the ability of apoptotic HL-60 cells to internalize transferrin was observed (Figure 10). Cells treated for 4 h with etoposide showed reduction in transferrin uptake of 70–80%, despite a reduction of only ~15–20% in levels of transferrin receptors in the same cells (Figure 8). The reduction in transferrin uptake was largely prevented by Ac-ZVAD.CH₂F. In the same sample of cells, ~80–90% of Rabaptin-5 was cleaved (see Figure 8). Much of the residual uptake in transferrin could probably be accounted for by the 10–15% of cells within the culture that remained resistant to etoposide even after 6 h. Attempts to measure changes in fluid-phase uptake by accumulation of HRP were unsuccessful, due to the high levels of HRP bound to apoptotic cells at 4°C (data not

Fig. 4. Inhibition of endosome fusion and cleavage of Rabaptin-5 are accelerated by caspase-3. (A) *Xenopus* egg extract was incubated without (top panel) or with (bottom panel) purified caspase-3 (1 μ g/ml final). Both incubations were performed in the absence or presence of 2 μ M Ac-DEVD-CHO. At the indicated time samples were analysed for Rabaptin-5 content by Western blot, using radio-iodinated protein A. (B) Parallel samples from the same experiment were assayed for their ability to support endosome fusion. Values are means from two separate experiments using the same extract and are expressed as a percentage of the fusion activity obtained from samples containing 2 μ M Ac-DEVD-CHO.

shown). However, uptake of lucifer yellow was substantially impaired in apoptotic cells versus control cells (data not shown). In summary, these findings provide evidence that membrane organization in general, and the endocytic pathway in particular, are affected during apoptosis.

Discussion

Historically, changes in nuclear morphology and DNA fragmentation have been described as classical markers for apoptosis (Earnshaw, 1995). However, the pronounced morphological changes, including alterations in membrane structure, which can be seen in the cytoplasm of all apoptotic cells indicate that cytoplasmic changes are fundamentally important. This is underlined by findings

Fig. 5. Loss of fusion activity is due to inactivation of cytosolic factor(s). (A) Egg extracts were incubated for 5 h at 23°C, then Ac-DEVD-CHO (2 μ M) was added (apoptotic). Alternatively, extracts were incubated with Ac-DEVD-CHO added at $t = 0$ (non-apoptotic). The indicated mixtures of each extract were assayed for their ability to support vesicle fusion. (B) Apoptotic and non-apoptotic extracts were mixed in the proportions indicated, then assayed for their ability to support endosome fusion. Values are means of duplicates from two experiments, expressed as a percentage of the activity obtained using a non-apoptotic extract.

that anucleated cells (Jacobson *et al.*, 1994) and cytoplasmic extracts (Newmeyer *et al.*, 1994; Martin *et al.*, 1995a; Cosulich *et al.*, 1996) undergo apoptosis. In this report, we have described for the first time the alteration of a specific cytosolic process, namely inhibition of endosome fusion, during apoptosis. Inhibition of membrane fusion can be prevented by specific inhibitors of caspases and by the negative regulators of apoptosis, Bcl-2 and Bcl-x_L. Furthermore, we have identified Rabaptin-5, a protein essential for membrane fusion, as a novel substrate for caspases.

Rabaptin-5 was recently identified as an essential and rate-limiting component of endosome fusion (Stenmark *et al.*, 1995). It interacts with the GTP-bound form of the small GTPase Rab5 and it is proposed that it acts as a downstream effector. An additional Rab5-interacting factor, p60, has also been described (Horiuchi *et al.*, submitted). Human Rabaptin-5 consists of two extensive domains capable of forming coiled coils, linked by a short central region. Within this region there are two potential sites for cleavage by caspases. The first (DESD⁴³⁸F) is very similar to sites within PARP (DEVD²¹⁷G) (Lazebnik *et al.*, 1994) and DNA-PKcs (DEVD²⁷¹²N) (Song *et al.*, 1996). The second site (VGAD⁴⁴⁶S) is more closely related to the caspase-6 cleavage site in lamin A (VEID²³⁰N) (Takahashi *et al.*, 1996). The sensitivity of cleavage of Rabaptin-5 to inhibitors of caspases is similar to that of

Fig. 6. Cleavage of Rabaptin-5 is responsible for loss of fusion activity. **(A)** Apoptotic extracts were assayed for their ability to support endosomal vesicle fusion in the presence of increasing amounts of additional extracts that had been depleted with control antibody ($\square$) or anti-Rabaptin-5 antibody ($\blacktriangle$). Data are presented as fusion activity relative to that supported by non-apoptotic extract and values are means $\pm$ s.e.m. from three experiments (left). Recombinant Rabaptin-5 (100 nM) was assayed for its ability to restore fusion activity to apoptotic extracts, or apoptotic extracts supplemented with Rabaptin-5-depleted extract. Data are presented as fusion activity relative to that supported by non-apoptotic extract and values are means from two experiments (right). **(B)** Fusion activity supported by apoptotic extracts or non-apoptotic extracts was measured in the presence or absence of cytosolic fractions enriched in Rabaptin-5 and containing Rabaptin-5-associated proteins (30 nM Rabaptin-5). Values are relative to those produced using non-apoptotic extract and are means from two experiments. **(C)** Non-apoptotic (A) or apoptotic (B) extracts were supplemented with buffer, or with Rabaptin-5-enriched cytosolic fractions that had been depleted with control or Rabaptin-5 antibody, as indicated, then assayed for endosome fusion activity. **(D)** Extracts treated for 5 h at 4°C (lane 1), or at 23°C with (lane 2) or without (lane 3) 2 μ M Ac-DEVD-CHO were analysed by Western blot for p60 content.

PARP and DNA-PKcs, which are cleaved by a protease of the caspase-3/Ced-3 subtype. However, preliminary evidence using *in vitro*-translated Rabaptin-5 suggests that it is not a direct caspase-3 substrate (manuscript in preparation). This is consistent with our finding that purified recombinant caspase-3, when added to *Xenopus* egg extracts, did not cause immediate cleavage of Rabaptin-5. We are currently attempting to determine the precise site of cleavage in both *Xenopus* and human Rabaptin-5. However, the generation of a 50 kDa C-terminal fragment in *Xenopus* egg extracts is consistent with cleavage occurring at a conserved site in an exposed region between the coiled coil-forming domains of Rabaptin-5.

There is now strong evidence to support the view that highly selective proteolysis of key substrates by caspases is essential in bringing about cellular changes seen in apoptosis. The specific role of Rabaptin-5 cleavage and the inhibition of vesicle fusion in apoptosis remains to be fully elucidated. However, recent studies on the function of Rab5 indicate how Rabaptin-5 cleavage would affect the organization of the endocytic pathway. Rabaptin-5 interacts with Rab5-GTP and stabilizes this form of the protein by reducing the rate of GTP hydrolysis (Rybin *et al.*, 1996). The GTP-bound form of Rab5, stabilized by

Fig. 7. Cleavage of Rabaptin-5 in apoptotic CEM-C7 cells. CEM C7 cells were incubated with 5 μ M dexamethasone and incubated for the indicated times. Cell samples (10^6) were sedimented and lysed. After equalizing protein concentrations, samples were run on SDS-PAGE and analysed for Rabaptin-5 (upper panel) or tubulin (lower panel).

a mutation that decreases GTP hydrolysis, stimulates endosome fusion in cell-free assays (Stenmark *et al.*, 1994). When overexpressed in cells, wild-type Rab5 increases the rate of endocytosis and leads to formation of enlarged endosomes (Bucci *et al.*, 1992). Likewise,

overexpression of Rabaptin-5 influences endosome structure (Stenmark *et al.*, 1995). In contrast, a mutant of Rab5 that prevents GTP binding, and is therefore predominantly in the GDP-bound form, is unable to stimulate vesicle fusion and results in endosome fragmentation and inhibition of endocytosis (Bucci *et al.*, 1992; Stenmark *et al.*, 1994). Since Rab5 is thought to act via its interaction with Rabaptin-5 (Stenmark *et al.*, 1995), cleavage of Rabaptin-5 during apoptosis would cause changes similar to those produced by expression of the inactive, GDP-bound form of Rab5. Such a phenotype is likely to contribute in several ways to the isolation of an apoptotic cell within a tissue, and the phagocytosis of that cell by

Fig. 8. Cleavage of Rabaptin-5 in HL-60 cells. HL-60 cells were treated as indicated with etoposide (50 μ M) with or without 50 μ M Ac-ZVAD. CH_2F . Cell samples were treated as in Figure 6 and analysed for Rabaptin-5 (upper panel), tubulin (middle panel), or transferrin receptor (lower panel) content.

its neighbours. Inhibition of endocytic activity would impair uptake of specific ligands, block nutrient uptake, and affect cellular motility. In addition, fragmentation of the endosome, which may form part of a wider-scale disintegration of membrane structure, would contribute to the fragmentation of the cell. Although the structure of specific organelles apart from the nucleus in apoptotic cells has not been reported, the absence of a recognizable Golgi complex and general vacuolation of membranes are features of many apoptotic cells (Kerr *et al.*, 1972). Fragmentation of membrane compartments was found to be coincident with Rabaptin-5 cleavage when we employed two unrelated cellular models for apoptosis. We are currently investigating the specific role of Rabaptin-5 cleavage in endosome fragmentation.

Additionally, regulation of certain cell surface receptors is a crucial event for the recognition and phagocytosis of apoptotic cells and their fragments (Duvall *et al.*, 1985). Although surface expression of phosphatidylserine may play a role in this recognition (Martin *et al.*, 1995c), other receptors including integrins have also been implicated (Savill *et al.*, 1990). There are instances where exposure of proteins at the cell surface is controlled by modulating their endocytic/recycling rates (Kelly, 1993; Verhey *et al.*, 1995). It is also known that inhibition of endocytic transport during mitosis alters receptor distribution (Warren *et al.*, 1984). Moreover, apoptotic neutrophils show impairment of both endocytic and secretory functions (Whyte *et al.*, 1993). Decline in endosome fusion in this cell-free system provides additional evidence that such regulation might also occur during apoptosis. We have

Fig. 9. Morphology of apoptotic cells. (A) Control HL60 cell. Magnification, $\times 5600$. (B) Apoptotic cell after 4 h treatment with etoposide. Note the condensation of chromatin and extensive vacuolation of cytoplasmic organelles. Magnification, $\times 7200$. (C) Detail of control cell. A Golgi complex is clearly visible (lower left), as well as the double bilayer of the nuclear envelope. Magnification, $\times 20\,000$. (D) Detail of apoptotic cell. Areas of condensed chromatin are devoid of a limiting membrane. Membranes within the mitochondria are still visible. Magnification, $\times 16\,000$.

Fig. 10. Transferrin uptake is impaired in apoptotic cells. HL-60 cells were treated with (filled) or without (unfilled) etoposide for 4 h, with (triangles) or without (squares) Ac-ZVAD.CM₂F. Samples were incubated with acridinium-labelled transferrin for the indicated time at 37°C, then washed to remove surface transferrin. Cell-associated acridinium-transferrin was assayed. A background for each condition was obtained by binding transferrin at 4°C, and subtracted (~20 000 rlu).

also shown that reduced uptake of transferrin occurs in apoptotic cells.

It is possible that cleavage of other proteins will contribute to disruption of the endocytic pathway in apoptotic cells. For example, it has been demonstrated recently that Rho-GDI is cleaved in apoptotic cells (Na *et al.*, 1996). Activated Rho and Rac markedly reduce the rate of receptor-mediated endocytosis (Lamaze *et al.*, 1996). Additionally, a novel Rho protein has recently been described that influences endosome motility (Murphy *et al.*, 1996). Interestingly, activated Rho D opposes the effect of activated Rab5 on endosome size. Therefore, it is possible that inactivation of Rab5 and activation of Rho proteins would have complementary effects on endocytic trafficking and endosome dynamics.

There may be parallels between the changes in membrane structure that occur during apoptosis and the major reorganization of the cytoplasm that occurs during cell division. In mitosis, fragmentation of intracellular membranes is thought to play a role in the distribution of their components to the daughter cells (Warren, 1993). The principal means by which this occurs is through inhibition of membrane fusion, mediated by the phosphorylation of components, as yet unidentified, by cyclin-dependent protein kinases. Inhibition of fusion has been clearly demonstrated in the case of endosome fusion using cell-free systems similar to that used in this study (Tuomikoski *et al.*, 1989; Woodman *et al.*, 1993). By analogy, cleavage of components of the vesicle fusion apparatus during apoptosis and subsequent breakdown of the endosome, and possibly other membranes, may be required for the fragmentation of the cell into apoptotic bodies. The discovery of Rabaptin-5 as a novel caspase substrate provides further evidence for the critical role that cleavage of key substrates plays in the dramatic cellular reorganization associated with apoptosis.

Materials and methods

Preparation of *Xenopus* egg extracts and HeLa nuclei

Egg extracts were prepared at 4°C as 10 000 g supernatants supplemented with an ATP-regenerating system and 1% glycerol as described (Cosulich *et al.*, 1996). Incubations were initiated immediately after thawing the extracts at 23°C.

HeLa nuclei were prepared as described by Wood and Earnshaw (1990) and stored at -80°C in storage buffer (10 mM PIPES, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 µg/ml protease inhibitors, 50% glycerol). Immediately after thawing, nuclei were washed in nuclear buffer at 4°C (10 mM PIPES, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 10 µM cytochalasin, 1 µM leupeptin, 1 µM pepstatin, 1 µM aprotinin, 1 µM chymostatin) and added to the extracts at the start of the incubation at a concentration of 1000 nuclei/µl. At the times indicated, 10 µl samples were lysed in 90 µl lysis buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS, 200 µg/ml proteinase K) and incubated overnight at 52°C. Samples were then phenol-extracted, ethanol-precipitated and run on 1% agarose gels. Gels were then incubated in Tris-borate-EDTA buffer containing 300 µg/ml RNase and 0.5 µg/ml ethidium bromide.

Expression of proteins and purification

Full-length human Bcl-2 cDNA was subcloned into pVL1393 baculovirus vector (Invitrogen) and expressed in Sf21 cells. Lysates of Bcl-2-expressing and control cells were made as described (Newmeyer *et al.*, 1994). A human Bcl-x_L cDNA was subcloned into pth375 vector, expressed in *Escherichia coli* using standard techniques and purified on a nickel resin (Qiagen). Recombinant Rabaptin-5 was purified as described (Stenmark *et al.*, 1995). Fractions of bovine brain cytosol enriched for Rabaptin-5 were generated according to procedures described elsewhere (Horiuchi *et al.*, submitted). Briefly, a 30% ammonium sulfate precipitate was subjected to gel filtration, ion exchange, and affinity chromatography on Ni-NTA resin. Preparations used here contained ~20% Rabaptin-5, and were also highly enriched for p60 (Horiuchi *et al.*, submitted).

Western blotting and immunodepletion experiments

For Western blotting, antibodies to the C-terminal portion of Rabaptin-5 (Stenmark *et al.*, 1995), Rab5 (Bucci *et al.*, 1994), Rab-GDI (Ullrich *et al.*, 1994) and NSF (6E6; Tagaya *et al.*, 1993) were used as described previously. Rabbit anti-α-SNAP serum was raised against His₆-α-SNAP and purified by affinity chromatography. Anti-p60 antibody was raised against a p60-containing peptide (Horiuchi *et al.*, submitted) and then affinity-purified. HRP-conjugated secondary antibody was used and blots were visualized on film by ECL. Alternatively, blots were incubated with protein A (10 µCi/ml) and visualized by phosphorimager. For quantitative Western blotting of human transferrin receptor, incubation with monoclonal anti-transferrin receptor antibody (Zymed Laboratories) was followed by incubation with ³⁵S-labelled anti-mouse antibody (1 µCi/ml; Amersham, Bucks, UK). Immunodepletion of extracts and Rabaptin-5-enriched fractions using control or Rabaptin-5-specific antibodies was performed essentially as described (Stenmark *et al.*, 1995). Depletion of Rabaptin-5 was confirmed by Western blotting.

Endosomal vesicle fusion

Donor membranes were prepared by internalization of biotin-transferrin (50 µg/ml) into suspension HeLa cells followed by preparation of crude endosomal membranes (Woodman and Warren, 1989). Acceptor membranes were prepared after internalization of anti-transferrin antibody (Woodman and Warren, 1989). Donor (5 µl) and acceptor (7 µl) membranes were incubated with 3 µl of extract for 1 h at 25°C, then dissolved in immunoprecipitation buffer [IB; 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2% (w/v) Triton X-100, 0.5% (w/v) SDS] containing *Staphylococcus aureus* cells. Pellets were washed in IB containing 0.5% *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfate as detergent, then incubated overnight with acridinium-labelled streptavidin (3 ng; Molecular Light Technology, Cardiff) in the same buffer. After further washing, acridinium-streptavidin was detected as described (Weeks *et al.*, 1986). Data are expressed as relative luminescence units (rlu) and are means of duplicates from representative experiments unless stated otherwise.

Cell culture

CEM-C7 cells were grown in Optimum medium supplemented with 5% FCS. After treatment with 5 µM dexamethasone for the indicated times, cells were lysed in lysis buffer [1% (w/v) NP-40, 25 mM HEPES-KOH,

pH 8.0, 0.4 M KCl, 0.5 mM Na-EDTA, 8 mM Na-EGTA, 1 mM DTT, 250 mM NaF, 5 µg/ml aprotinin, 10 µg/ml pepstatin A] and analysed for proteins by Western blot. HL-60 cells were grown in RPMI 1640 medium with 5% FCS. Cells were induced to undergo apoptosis by treatment with 50 µM etoposide (Beere *et al.*, 1996). After the indicated times, equal numbers of cells were harvested by centrifugation. Samples were either solubilized in lysis buffer and analysed for proteins by Western blot, or used for transferrin uptake experiments.

Transferrin uptake

Human transferrin (100 µg) was labelled with succinimidyl acridinium ester (Molecular Light Technologies) according to the manufacturer's instructions to obtain a final activity of $\sim 10^9$ rlu/µg. HL-60 cells ($\sim 10^6$ per sample) were washed three times in PBS, then resuspended in 100 µl binding medium [CO_2 -independent medium (Gibco) containing 0.2% (w/v) BSA]. After warming each sample to 37°C, acridinium-transferrin (10 µg/ml) was added and the incubation continued for the time shown. Samples were washed three times in ice-cold PBS, once over 15 min in ice-cold pH 5 saline (100 mM NaCl, 50 mM Na citrate, pH 5.0) containing 0.1% BSA, then once over 15 min in PBS containing 0.1% BSA. Pellets were resuspended in PBS containing 1% Triton X-100 and, after removing debris by centrifugation, samples were assayed for acridinium content. For each experiment, labelled transferrin was added to a control sample which was incubated for 60 min at 4°C.

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