Human Rabaptin-5 Is Selectively Cleaved by Caspase-3 during Apoptosis*

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We have previously shown that Xenopus rabaptin-5 is cleaved in apoptotic extracts, with a concomitant reduction in the ability of these extracts to support endosomal membrane fusion (Cosulich, S. C., Horiuchi, H., Zerial, M., Clarke, P. R., and Woodman, P. G. (1997) EMBO J. 16, 6182–6191). In this report we demonstrate that caspase-dependent cleavage is a conserved feature of rabaptin-5. Human rabaptin-5 is cleaved at two sites (HSLD and DESD) in apoptotic HeLa extracts. Cleavage is effected by caspase-3, since it is prevented when caspase-3 activity is either inhibited by Ac-DEVD-CHO or removed by immunodepletion. Moreover, an identical pattern of cleavage is observed using recombinant caspase-3. The action of caspase-3 is highly selective; neither caspase-2 nor caspase-7 are able to cleave recombinant or cytosolic rabaptin-5. Caspase-dependent cleavage of rabaptin-5 generates two physically separated coiled-coil forming domains, the C-terminal of which retains the ability to bind the Rab5 exchange factor rabex-5.

Programmed cell death (apoptosis) plays a fundamental role in the development and homeostasis of multicellular organisms (1, 2). The primary feature of apoptosis is rapid engulfment and degradation of dying cells by their neighbors, so that an inflammatory response can be avoided. Since in many cases the engulfing cells are not specialized for phagocytic uptake (3), signals that expedite engulfment and degradation are likely to arise from the apoptotic cell. A critical event during apoptosis is therefore the expression of surface receptors that permit the specific recognition of a dying cell. One such receptor is probably phosphatidylserine, which is translocated from the inner leaflet to the outer leaflet of the plasma membrane during apoptosis (4). There is considerable evidence, however, that other surface moieties, including carbohydrate, form part of the recognition signal (5).

In addition to changes at the surface, the changes in cellular function that occur in an apoptotic cell are characterized by a variety of striking morphological and biochemical alterations. These include fragmentation of the nucleus and activation of endonuclease(s) (6, 7), cell shrinkage and fragmentation, and plasma membrane blebbing (8). A further distinguishing feature of apoptotic cells is a loss of organized endomembrane structure; the nuclear envelope is frequently lost, and other recognizable membrane structures such as the Golgi complex are replaced by a disorganized array of vacuoles and vesicles (9, 10). The so-called execution phase of apoptosis is evolutionarily conserved (11), underlining its importance.

It is now widely believed that many (although not all) apoptotic changes are linked to activation of a number of conserved cysteine proteases (caspases), which cleave specific substrates involved in key cellular processes (for review see Ref. 12). Caspases themselves are present as proenzymes that are readily cleaved, either autocatalytically (13, 14) or by upstream "activator" caspases (15, 16), during apoptosis. This provides the cell with a means to rapidly amplify its apoptotic response. Caspases can be divided into groups based on their sequence-selective protease activity toward peptide substrates. Thus, caspases-2, -3, and -7 (group II caspases) all cleave preferentially after the sequence XEXD (17). A major question is whether such overlapping substrate specificity within each group indicates that these enzymes represent tissue-specific isoforms or redundant isoforms within the same cell, or whether caspases exhibit far greater specificity toward polypeptide substrates in vivo.

Given the profound morphological changes occurring to membranes within apoptotic cells, and evidence for alterations in the expression of surface receptors, we anticipated that apoptosis would be associated with changes in the dynamics of the endocytic/recycling pathways. On this basis, we examined whether endosomal membrane fusion, an event that is essential for endosomal organization and for transport of receptors through the endocytic recycling pathway, is affected in apoptotic extracts. Endosomal fusion was indeed substantially reduced during apoptosis in Xenopus extracts, and this reduction was associated with specific cleavage of the Rab5 effector rabaptin-5 (18). Cleavage of rabaptin-5 was also apparent in cellular models of apoptosis, and was accompanied by reduced endocytic capacity. Hence, rabaptin-5 cleavage appears to be an important determinant in the abrogation of normal cellular function during apoptosis.

In this study, we have examined in detail the activity that cleaves rabaptin-5. We have also mapped the site of rabaptin-5 cleavage, in order to understand how it might interfere with rabaptin-5 function and thus contribute to impairment of normal endocytic transport. Our previous data suggested that rabaptin-5 is cleaved by a caspase-related activity (18). We now show that caspase-3 cleaves human rabaptin-5 at two closely positioned and conserved sites to generate physically separated N- and C-terminal domains. The activity of caspase-3 toward rabaptin-5 is surprisingly selective, since neither caspase-2 nor caspase-7 effect cleavage.

EXPERIMENTAL PROCEDURES

Reagents—Cytochrome c was obtained from Roche Molecular Biochemicals Ltd., Lewes, Sussex, United Kingdom (UK). Ac-DEVD-CHO1

1 The abbreviations used are: CHO, Chinese hamster ovary; AMC,aminomethylcoumarin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PARP, poly(ADP)-ribose polymerase.

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and Ac-DEVD-AMC were bought from Calbiochem-Novabiochem (UK) Ltd. (Nottingham, UK) and stored as 10 mM stocks in Me2SO at −20 °C. Ac-LDEND-AMC was synthesized by SNPE Ltd. (Croyden, Surrey, UK). Antibodies to caspase-3 (N-19, H-277) were from Santa Cruz Inc. (Autenbioclear, Calif., Wils, UK). Antiserum to EEA1 was a kind gift from Dr. E. Webb (The Rockefeller University, New York, NY). Antibodies and reagents to rabaptin-5 and rubex-5 were generously provided by Marino Zerial, Max Planck Institute for Molecular Cell Biology and Genetics, c/o EMBL, Heidelberg, Germany. Recombinant active caspases were generous gifts from Donald Nicholson and Sophie Roy, Merck Frosst Center for Therapeutic Research, Quebec, Canada. Rabbit peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). For ECL Western blotting were obtained from Dako, Glostrup, Denmark. All other reagents were obtained from Sigma.

**RESULTS**

**Human Rabaptin-5 Is Cleaved during Apoptosis**—Our previous work had demonstrated that *Xenopus* rabaptin-5 is cleaved in egg extracts to yield a C-terminal fragment of 45–50 kDa (18). Similar extracts have been shown to undergo several apoptotic events, including activation of caspases and endonucleases (26, 27). To establish whether cleavage by apoptotic proteases is a conserved feature of rabaptin-5, human His6-rabaptin-5 was translated *in vitro* and then combined with a *Xenopus* egg extract. After appropriate incubation, rabaptin-5 cleavage products of approximately 62 (Fig. 1A) and 47 kDa (Fig. 1B) were produced, which could be immunoprecipitated by anti-His antibody (Fig. 1A) or an antibody recognizing the C-terminal portion of rabaptin-5 (Fig. 1B), respectively. The time course of cleavage was similar to that previously reported for cleavage of endogenous *Xenopus* rabaptin-5 (18) (data not shown), and was prevented by inclusion of the specific caspase inhibitor Ac-DEVD-CHO (Fig. 1). Hence, human His6-rabaptin-5 behaves in apoptotic *Xenopus* extracts in the same way as *Xenopus* rabaptin-5, confirming that cleavage is a conserved function.

Recent work from Wang and colleagues (16, 28, 29) has shown that cytosolic extracts from mammalian cells can be triggered to enter apoptosis by addition of cytochrome c. This process can be sensitized by, although it is not strictly dependent upon, inclusion of dATP (24, 28). We examined whether this system would be convenient for studying the apoptotic cleavage of rabaptin-5. Previous work has shown that activation of caspase-3 in HeLa extracts occurs via cytochrome c-dependent activation of caspase-9 (16), and is maximal within 30 min of addition of cytochrome c (24). Substrates for caspase-3 and/or downstream caspases are cleaved more slowly. For example, caspase-2, whose cleavage requires caspase-3 activity (24, 30), is cleaved in these extracts between 1 and 3 h after addition of cytochrome c to yield an immunoreactive 12/13-kDa fragment (24). Rabaptin-5 is also cleaved, although somewhat more slowly than caspase-2, to yield a C-terminal fragment similar in size to that generated within apoptotic *Xenopus* extracts. Using a monoclonal antibody that recognizes an epitope within the N-terminal portion of rabaptin-5, the 62-kDa N-terminal fragment was also identified (Fig. 2B). An additional minor product of approximately 53 kDa (N-53) was also seen with this antibody, suggesting the presence of a further cleavage site. This was confirmed by inclusion of *in vitro* translated rabaptin-5 (see Fig. 3). Prolonged incubation of apoptotic extracts resulted in the disappearance of the 62-kDa fragment, while the N-53 fragment remained resistant to further proteolysis (Fig. 2C, left panel), consistent with it being the result of a second, slower, cleavage event. Importantly, appearance of a 53-kDa
A DESD438F. Asp438 rabaptin-5 still gave rise to the N-53 frag-
ment, confirming that it is the product of a second cleavage
event that is not dependent on prior cleavage at Asp438.

Polypeptide reactive against this antibody was observed in
apoptotic cells and correlated with the disappearance of full-
length rabaptin-5 (Fig. 2C, right panel). These results suggest
that N-53 is a final cleavage product in apoptotic cells. More-
over, they demonstrate that the cleaving activity that is pres-
ent in apoptotic cells is likely to be similar, if not identical, to
that present in cytochrome c-activated extracts.

The amino acid sequence of rabaptin-5 (23) and the size of
the C-terminal cleavage product in Xenopus extracts (18) had
suggested to us that caspase-dependent cleavage was most
likely to occur after aspartate 438 or aspartate 446. Indeed,
inclusion of a 22-mer peptide that included both aspartate
residues retarded cleavage in Xenopus extracts by 1–2 h (data
not shown). To identify the exact cleavage site, rabaptin-5
mutants were prepared with either Asp438 or Asp446 replaced
by alanine, and were incubated in apoptotic HeLa extracts.
Compared with wild-type rabaptin-5 (Fig. 3), HeLa cytosol was
incubated for 5 h at 30 °C without (control) or with (apoptotic) 10 μM cytochrome
c, then analyzed by Western blot with a rabaptin-5 monoclonal antibody
which recognizes the N terminus of the protein. C. HeLa cytosol was
incubated for 0 or 16 h at 30 °C, then analyzed by Western blot using
anti-rabaptin-5 N-terminal antibody (left panel). HL60 cells were left
untreated, incubated with 50 μM etoposide for 5 h or with 1 μg/ml
anisomycin for 3 h, then isolated by centrifugation and lysed in SDS-
PAGE buffer. Equivalent amounts were applied to SDS-PAGE and
analyzed by Western blot using anti-rabaptin-5 N-terminal antibody
(right panel).

FIG. 2. Rabaptin-5 is cleaved in apoptotic HeLa extracts. A, HeLa cytosol was incubated at 30 °C in the presence of 10 μM cyto-
chrome c for the indicated times, then analyzed by Western blot for
rabaptin-5 (anti-C-terminal antibody). B, HeLa cytosol was incubated
for 5 h at 30 °C without (control) or with (apoptotic) 10 μM cytochrome
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Studies of caspase cleavage sites using synthetic modified tet-
rapeptides have identified the amino acid preferences at the
P2–P4 positions for each caspase (17). Based on these studies,
caspases have been divided into several groups. The group II
“effector” caspases, caspase-3 and caspase-7, both cleave effec-
tively after the sequence DEVD. Although this is similar to the
primary cleavage site within rabaptin-5 (DES), the activity of
both caspases toward peptides is reduced when serine is placed
at P2. In contrast, DESD is a preferred cleavage site, second
only to DEHD, for the group II “activator” caspase, caspase-2
(17). Based on these studies, and our previous observation that
concentrations of recombinant caspase-3 just sufficient to ac-
celerate apoptotic changes in Xenopus egg extracts were unable
to cleave rabaptin-5 directly (18), it seemed likely that a
caspase-2-like activity would be responsible for cleaving rabap-
tin-5. However, we undertook a detailed examination to estab-
lish the true identity of the cleaving activity.

First, we examined whether cleavage of cytosolic rabaptin-5
in HeLa extracts is dependent on caspase-3 activity. We have
already used this approach to establish that cleavage of
caspase-2 occurs via caspase-3 (24). We first examined the
sensitivity of cleavage to the specific caspase-3 inhibitor Ac-
DEVD-CHO, and found that cleavage of rabaptin-5 was pre-
vented by inclusion of 50 nM Ac-DEVD-CHO (data not shown),
similar to those concentrations that prevent cleavage of the
caspase-3 substrates PARP and caspase-2 (24). To further es-
establish a dependence on caspase-3 activity, extracts were de-
pleted of caspase-3 prior to addition of cytochrome c. Cytosols
pre-treated with an antibody to caspase-3 were depleted of
caspase-3 precursor by at least 90% compared with mock-de-
depleted cytosols (data not shown). When incubated with cyto-
chrome c, these extracts were unable to cleave rabaptin-5
(Fig. 4A, left panel). In contrast, rabaptin-5 was cleaved almost
to completion within 4 h when mock-depleted extracts were

Rabaptin-5 Is Cleaved Selectively by Caspase-3—Systematic
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to completion within 4 h when mock-depleted extracts were
incubated with cytochrome c (Fig. 4A, right panel).

Although cleavage of cytosolic rabaptin-5 is dependent on caspase-3, it is possible that it is cleaved directly by a downstream effector of caspase-3, such as caspase-2. To address this question, HeLa extracts were preincubated for a period sufficient to activate caspase-3, as well as potential downstream caspases. They were then depleted of activated caspase-3. Western blotting of extracts confirmed that greater than 90% of activated caspase-3 had been removed (data not shown). When excess bacterially expressed rabaptin-5 was added to depleted extract, no cleavage product was detected before 3 h, and significant cleavage did not occur until after 5 h incubation (Fig. 4B, top left). In contrast, cleavage of rabaptin-5 was observed within 1 h of its addition to mock-depleted extract, and the majority of recombinant rabaptin-5 was cleaved after 4–5 h (Fig. 4B, top right). The rate at which caspase-3-depleted extract could cleave rabaptin-5 was increased significantly by inclusion of recombinant caspase-3 (Fig. 4B, bottom left) or the beads isolated from the immunodepletion step (Fig. 4B, bottom right), confirming that the major cleaving activity was caspase-3.

To demonstrate that the caspase activated within apoptotic cells most likely to cleave rabaptin-5 is caspase-3, cytosol was prepared from apoptotic HL60 cells. These cytosols had substantial rabaptin-5 cleaving activity, since significant cleavage of recombinant rabaptin-5 was observed within 1–2 h (Fig. 4C, left panel). Again, prior depletion of active caspase-3 from these extracts significantly reduced the rate at which rabaptin-5 was cleaved (Fig. 4C, right panel).

These results indicated that the major rabaptin-5 cleaving activity within apoptotic extracts is caspase-3. To confirm this, and to further demonstrate the selectivity of rabaptin-5 as a caspase-3 substrate, recombinant rabaptin-5 was incubated with purified caspases. As shown in Fig. 5A (left panel), rabaptin-5 was cleaved in an Ac-DEVD-CHO-sensitive manner when incubated with immunoprecipitated activated caspase-3. Furthermore, recombinant caspase-3 cleaved rabaptin-5 to generate the same cleavage products as did apoptotic cytoso (Fig. 5A, right panel). Cleavage of rabaptin-5 was first observed at caspase-3 concentrations of 0.5 nM or above (Fig. 5B). In contrast, PARP cleavage was observed above 0.1 nM caspase-3 (Fig. 5C) and caspase-2 cleavage was observed above 0.25 nM caspase-3 (Fig. 5D). This somewhat lower activity of caspase-3 toward rabaptin-5 compared with caspase-2 correlates well with the slower rate of rabaptin-5 cleavage in apoptotic extracts. By analyzing caspase-3 cleavage of purified in vitro translated rabaptin-5 at 37 °C (data not shown) we obtained a $K_{cat}/K_{m}$ of $1 \times 10^6 \text{m}^{-1} \text{s}^{-1}$ (versus PARP; $20 \times 10^6 \text{M}^{-1} \text{s}^{-1}$).

Caspase-2 has a preference for the cleavage site DESD over DEVD, so it was expected that rabaptin-5 would be a good
Caspase-3 Cleaves Rabaptin-5

**Fig. 5.** Rabaptin-5 is cleaved by caspase-3, but not by caspase-2 or caspase-7. **A**, left panel, apoptotic HeLa cytosols were incubated with protein G-agarose beads coated with control or anti-caspase-3 antibody. After washing, the beads were incubated as indicated with in vitro translated His$_6$-rabaptin-5, and the products analyzed by SDS-PAGE and phosphorimaging. **Right panel**, in vitro translated His$_6$-rabaptin-5 was incubated with buffer, with recombinant caspase-3 (1 nM), or with HeLa cytosol preactivated by incubating with cytochrome c. **B**, in vitro translated His$_6$-rabaptin-5 was incubated with recombinant caspase-3 as indicated. **C**, as **B**, but with in vitro translated PARP. **D**, in vitro translated His$_6$-rabaptin-5 was incubated with recombinant caspase-2 as indicated. **E**, as **D**, but with in vitro translated caspase-3. **F**, in vitro translated His$_6$-rabaptin-5 was incubated with recombinant caspase-2 as indicated. **G**, as **F**, but with in vitro translated caspase-3. **H**, in vitro translated His$_6$-rabaptin-5 was incubated with recombinant caspase-7 as indicated. **I**, as **G**, but with in vitro translated PARP.

Caspase-3 substrate for caspase-2 (17). Surprisingly, no cleavage of rabaptin-5 was observed at concentrations of recombinant caspase-2 as high as 2 $\mu$M (Fig. 5E). The activity of the caspase-2 preparation was confirmed by examining its ability to cleave caspase-3, which was observed above 0.5 nM recombinant caspase-2 (Fig. 5F). Importantly, no self-cleavage by in vitro translated caspase-3 was observed. Likewise, the activity of caspase-2 and its preference for the sequence DESD over DEVD (17) was confirmed by measuring the activity of recombinant caspase-2 toward fluorogenic substrates (Ac-DEVD-AMC: $K_m = 47$ $\mu$M; $V_{\text{max}} = 1344$ fluorescence units/min/nmol; Ac-DEVD-AMC: $K_m = 107$ $\mu$M; $V_{\text{max}} = 171$ fluorescence units/min/nmol).

Studies so far have suggested that the substrate specificity of caspase-3 and caspase-7 are very similar (see Ref. 12 for review), tempting speculation that they may be redundant activities. However, we observed no cleavage of rabaptin-5 by recombinant caspase-7, even at concentrations as high as 580 nM (Fig. 5G). Again, the activity of the caspase-7 preparation was confirmed by examining its ability to cleave PARP (Fig. 5H) or Ac-DEVD-AMC ($K_m = 27$ $\mu$M; $V_{\text{max}} = 1608$ fluorescence units/min/nmol).

The activity of recombinant caspase-3 toward rabaptin-5 was exploited to obtain the N-terminal sequences of the major cleavage fragments. Recombinant rabaptin-5, tagged at the C terminus with a peptide derived from protein C, was incubated with caspase-3 before being immunoprecipitated with anti-protein C beads. When the cleavage products were eluted and sequenced, the C-47 fragment was found to contain the N-terminal sequence FGFLVGADSV. Since this sequence corresponds exactly to that starting at amino acid 439 within full-length rabaptin-5, this confirms that the primary caspase-3 cleavage site is after DESD$^{439}$. A higher molecular mass product of approximately 54 kDa gave the N-terminal sequence AGL(-)(-)PS-Pro-Lys-Glu-$\mathcal{X}$ (where $\mathcal{X}$ was not resolved), corresponding to the sequence immediately after HSLD$^{379}$. The slower cleavage observed at this site within extracts is consistent with it being further removed from DESD from the optimal caspase-3 cleavage site.

Although the data presented above are consistent with selective cleavage of rabaptin-5 by caspase-3, it remained possible that the same specificity would not be observed for cytosolic rabaptin-5. This point seemed particularly important, given that cytosolic rabaptin-5 is found as part of a large complex, which also includes the Rab5 guanine nucleotide exchange factor rabex-5 (31). Components of this complex might influence caspase specificity by influencing the orientation or exposure of the cleavage sites within rabaptin-5, or by recruiting alternative caspases. To address this issue, cytosolic rabaptin-5 was fractionated away from endogenous caspase-2 and caspase-3 by gel filtration chromatography. Its ability to act as a substrate for caspase-2 or caspase-3 was then compared with that of recombinant rabaptin-5. As shown in Fig. 6 (upper panels), cleavage of both cytosolic and recombinant rabaptin-5 was first observed at the same concentration of caspase-3 (0.5–1 nM). Similarly, neither preparation of rabaptin-5 was cleaved by caspase-2 concentrations as high as 2 $\mu$M (Fig. 6, lower panels). For these experiments rabaptin-5 was added well in excess, so that product formation would be linear in relation to enzymic activity.

Rabaptin-5 Cleavage Separates N- and C-terminal Fragments—Rabaptin-5 forms part of a high molecular weight complex containing rabex-5 (31), and has been shown to bind to a number of effectors including Rab5 (23) and Rab4 (32). We sought to establish, therefore, whether caspase-dependent cleavage of rabaptin-5 would give rise to physically separate subcomplexes and thus potentially provide a means to uncouple the action of various effectors. This was investigated first by
co-immunoprecipitation using antibodies specific for either N- or C-terminal fragments of rabaptin-5. In control extracts, immunodepletion of rabaptin-5 from cytosol using an anti-N-terminal antibody led to complete loss of rabaptin-5, as detected both by anti-N-terminal and anti-C-terminal antibodies, as expected (Fig. 7A). Likewise, immunodepletion with anti-C-terminal antibody led to complete loss of anti-N- and anti-C-terminal reactivity. When apoptotic extracts were depleted with anti-N-terminal antibody, complete loss of anti-N-terminal reactivity was observed. However, significant amounts of the C-47 fragment remained. Conversely, immunoprecipitation with anti-C-terminal antibody led to complete depletion of this fragment, while the N-terminal fragment remained in the supernatant.

The fate of the rabaptin-5 fragments was also followed by gel filtration chromatography (Fig. 7B). When apoptotic cytosol was applied to a Superose 6 column, residual full-length rabaptin-5 migrated in fraction 6, and could be detected with both anti-N- and anti-C-terminal antibodies. Rabaptin-5 from control extracts migrated to a similar position (data not shown). The band in fraction 8 that cross-reacted with the anti-C-terminal antibody was identified as rabaptin-5β, a rabaptin-5-related protein (33), by the use of an antibody specific for rabaptin-5β (data not shown). The rabaptin-5 fragments migrated as distinct but overlapping lower molecular weight species. Both N-62 and N-53 fragments migrated to fraction 9, and to some extent to fraction 10 (Fig. 7B, top panel). The C-47 fragment migrated slightly more slowly, with most appearing in fraction 10 and some in fraction 11 (Fig. 7B, middle panel). Thus, caspase cleavage results in physical separation of the two halves of rabaptin-5 into lower molecular weight complexes.

Physical separation of the rabaptin-5 fragments allowed us to determine which portion of the protein binds to rabex-5. When fractions from the Superose 6 column were analyzed with antibodies to rabex-5, two peaks of reactivity were observed (Fig. 7B, bottom panel). The first peak was found in fraction 8, coincident with rabaptin-5β, which is known to bind directly to rabex-5 (31, 33). The second peak was found in fraction 10, with the bulk of the C-47 reactivity. Although this suggested that rabex-5 interacted with the C-terminal portion of rabaptin-5, the data were complicated by the presence of rabaptin-5β. To further examine the binding of rabex-5, co-immunoprecipitation of rabex-5 with the anti-N-terminal rabaptin-5 antibody was measured. As shown in Fig. 7C, rabex-5 co-immunoprecipitated with anti-rabaptin-5 N-terminal antibody in control cytosol. However, efficient co-immunoprecipitation was not observed after rabaptin-5 was cleaved in apoptotic extracts. This provides evidence that the C-terminal portion of rabaptin-5 is required for efficient rabex-5 binding. The converse experiment, of demonstrating that rabex-5 could
Caspase-3 Cleaves Rabaptin-5

The ability of HeLa cytosol to support endosome fusion was reduced with increasing time in the presence of cytochrome c, and this reduction was accompanied by cleavage of rabaptin-5 (Fig. 8, A and B). Inhibition of fusion was only partial and, in contrast to previous studies using Xenopus egg extracts (18), could be overcome to some extent by increasing the cytosol concentration (data not shown). This was most likely due to the resistance of rabaptin-5β to apoptotic cleavage (data not shown). In any case, it did not allow for reconstitution experiments to demonstrate whether cleavage of rabaptin-5, or other effectors of fusion, is solely responsible for the reduction in fusion activity, as appears to be the case in Xenopus egg extracts. Cleavage of rabaptin-5 remains the most likely cause of the reduction in fusion activity, since rabex-5 is not cleaved in apoptotic extracts and the Rab5 effector EEA1 (8, 34) concentration is reduced only slightly (data not shown). However, cleavage of other effectors of endosome fusion cannot be ruled out.

DISCUSSION

In this report we have investigated the caspase-specific cleavage of rabaptin-5 in cytochrome c-activated human cell extracts. We have demonstrated directly and by mutagenesis experiments (for one site only) that rabaptin-5 is cleaved at two sites: cleavage in extracts occurs more rapidly at the sequence DESD438, but also occurs at HSLD479. Cleavage of rabaptin-5 at this second site is followed to completion in apoptotic cells, most likely by caspase-3, since examination of apoptotic cells revealed a stable N-terminal product co-migrating precisely with rabaptin-5 1-379. Further data indicate that caspase-3 is responsible for rabaptin-5 cleavage in apoptotic cells, since immunodepletion of caspase-3 substantially reduced rabaptin-5 cleaving activity from extracts made directly from apoptotic cells.

Both caspase cleavage sites are conserved between all rabaptin-5 sequences currently on the data base. Therefore, it is probable that the cleavage of Xenopus rabaptin-5 that we previously observed corresponded to that at DESD438, underlining the fact that caspase-dependent cleavage is likely to be a conserved feature of this protein. The absence of cleavage at HSLD479 in Xenopus extracts may reflect somewhat lower caspase activity, or a slightly different substrate specificity of Xenopus versus human caspase-3. Analysis of rabaptin-5 reveals two extensive domains capable of forming coiled coils, which generate a rodlike parallel homodimer (23). Between the N- and C-terminal helical domains is a non-helical linker region (approximately amino acids 350–530). Thus, cleavage at Asp479 and Asp438 would be expected to cause separation of these domains, at least in the purified protein. Despite the fact that it forms part of a high molecular weight complex (23), our data indicate that physical separation of these domains also occurs within cytosolic rabaptin-5.

Rabaptin-5 has previously been identified as a downstream Rab5 effector, which is essential for endosome fusion and which binds Rab5 within its C-terminal domain (23, 32). Rab5 is a member of the family of small GTP-binding proteins that participate in intracellular membrane docking/fusion reactions (35). It cycles between a cytosolic GDP-bound pool and a membrane-associated GTP-bound form (36). It is in the latter conformation that Rab5 is active and able to recruit effectors of endosome fusion, including rabaptin-5 and EEA1 (23, 37, 38). The precise role that Rab effectors such as these play in membrane docking/fusion is not clear, although evidence indicates that they participate in peripheral “tethering” of membranes (39) and may in addition activate the appropriate docking receptors (SNAREs (soluble N-ethylmaleimide factor attachment protein receptor)) within opposing membranes (40, 41).

Recent reports demonstrate that the role of rabaptin-5 during endosome fusion appears more complex than simply that of a downstream effector, however. First, rabaptin-5 itself reduces the GTPase activity of Rab5 and will thus maintain Rab5 in its active form (42). Second, rabaptin-5 forms a cytosolic complex with a Rab5 guanine nucleotide exchange factor, rabex-5 (31). Hence, recruitment of rabaptin-5 by Rab5-GTP may enhance localized exchange activity. Since rabaptin-5 forms a homodimer (32) and could thus recruit two molecules of rabex-5 per Rab5 monomer, this may provide a means to focus to within the membrane where Rab5 activity is retained (31). To date, the site within rabaptin-5 that recruits rabex-5 has not been identified. However, our studies provide strong indications that rabex-5 binds to the C-terminal portion of rabaptin-5, thereby placing Rab5 exchange activity adjacent to Rab5 itself. Our findings therefore indicate that inhibition of endosome fusion in apoptotic extracts is unlikely to be due simply to an inability to maintain Rab5 in its active conformation. It is more likely that cleavage of rabaptin-5 prevents the recruitment of further Rab5 effectors. Indeed, the N-terminal portion of rabaptin-5 behaves as a higher molecular weight species than the C-terminal rabaptin-5/rabex-5 complex, indicating that it is bound to other cytosolic component(s).

Intriguingly, rabaptin-5 will also bind, via its N terminus, Rab4 (32). Rab4, like Rab5, is localized to the early endosome, though the activity of Rab4 seems to oppose that of Rab5. Thus, Rab4 is apparently required for a transport pathway that leads away from the early endosome, most likely the recycling of vesicles to the cell surface (43). The finding that rabaptin-5 will bind Rab proteins involved in both endocytic and exocytic pathways raises the possibility that it acts as a functional linker,
co-ordinating the fluxes of both pathways (32). In this way, the ratio of external to internal membrane can be maintained despite the rapid movement of material between these compartments. The notion that rabaptin-5 acts as a link between endocytic and exocytic pathways is further supported by the finding that rabaptin-5 interacts with the Rab3 effector rabphilin-3 (44). Cleavage of rabaptin-5 by apoptotic proteases will destroy this functional linkage, and may contribute to the changes in membrane dynamics and morphology that are apparent in apoptotic cells. A full explanation of the role of rabaptin-5 cleavage in reorganization of endosomal membranes, and possible compensating effects of other interacting proteins such as rabaptin-5γ, must await examination of these membranes in individual cells undergoing apoptosis.

We have shown that rabaptin-5 is cleaved, both at Asp379 and Asp383, by caspase-3. Cleavage occurs above 0.5 nM caspase-3, somewhat higher than the concentration required to cleave PARP or caspase-2, two well characterized substrates for caspase-3 (45), and is consistent with the 2–3-fold slower rate of cleavage of rabaptin-5 compared with caspase-2 in HeLa extracts. The $K_{cat}/K_{m}$ is however similar to that for other identified caspase-3 substrates, for example focal adhesion kinase (46) and huntingtin (47), as well as being very close to that of caspase-1/ interleukin-1β-converting enzyme for its cellular substrate, interleukin-1β (47, 48). We expected other group II caspases (17) to cleave rabaptin-5, particularly at DESD438. However, neither the “effector” caspase, caspase-7, nor the “activator” caspase, caspase-2, cleaved rabaptin-5 when used at concentrations 100-fold or greater than caspase-3. These data are fully consistent with our finding that the major cleaving activity in apoptotic cells is caspase-3. Crucially, our results rule out the possibility that cytosolic binding partners might influence the specificity of rabaptin-5 cleavage either by changing the conformation of the substrate or by recruiting other caspases. Although it is possible that membrane-bound rabaptin-5 has an altered caspase susceptibility, our preliminary observations indicate that addition of membrane does not influence the pattern or rate of rabaptin-5 cleavage (data not shown).

The inability of caspase-2 or caspase-7 to cleave rabaptin-5 is particularly surprising, given that several polypeptide substrates are cleaved efficiently by all group II caspases (see Ref. 12 for review). In particular, the discrepancy between caspase-2 and -3 is odds with our kinetic data using peptide substrates, where both caspases cleave LDDESD effectively. Our finding demonstrates the importance of the tertiary structure of substrates in determining caspase recognition. It further supports the hypothesis that caspases with apparently similar protease activities fulfill selective roles within the apoptotic cell, rather than being merely redundant activities. In the case of caspase-2, such selectivity is consistent with its role as an “activator” caspase, which acts as a functional linker between apoptotic stimuli and downstream “effector” caspases. The inability of caspase-7 to cleave rabaptin-5 is more surprising. Several studies have demonstrated how selectivity of caspase activation may be achieved by differential localization of caspases in apoptotic cells (49, 50). This is among the first instances where such selectivity appears to be a consequence of biochemical specificity.