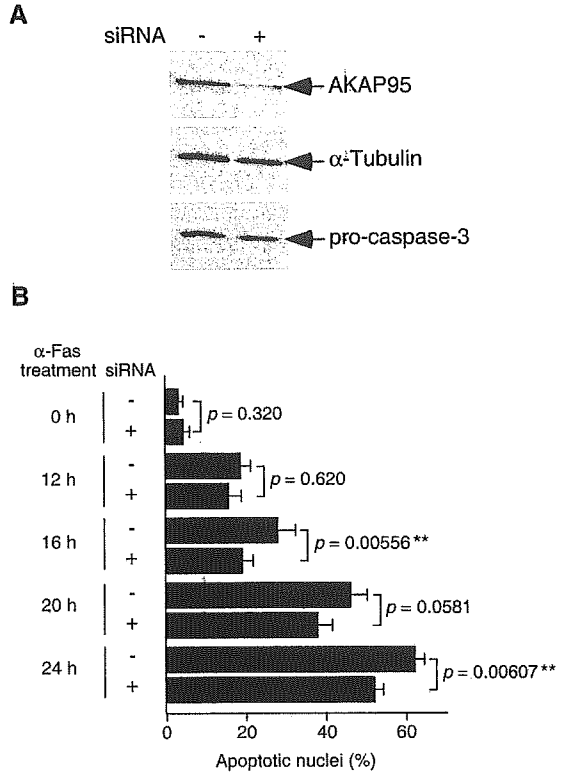

FIG. 2. Inhibition of nuclear morphological changes of apoptotic cells by siRNA for AKAP95. (A) Lysates from HepG2 cells transfected with or without siRNA for AKAP95 for 4 days were fractionated by SDS-PAGE and immunoblotted with anti-AKAP95 polyclonal antibodies, anti-α-tubulin monoclonal antibody, or anti-caspase 3 monoclonal antibody. (B) HepG2 cells transfected with or without siRNA for AKAP95 were treated with an agonistic anti-Fas antibody in the presence of actinomycin D for the indicated periods and collected and fixed with 3.7% formaldehyde for 10 min. After staining with Hoechst 33342, the percentage of the cells showing apoptotic nuclei to the total cells was measured. At least 100 cells were counted for each measurement in all experiments. The data (mean ± the standard deviation) were obtained from at least three independent experiments. Significant test results (P values) are shown. **, $P < 0.01$.

anti-AKAP95 monoclonal (Fig. 1, upper panel) or polyclonal (Fig. 1, middle panel) antibodies during apoptosis. These results suggested that AKAP95 is probably not a substrate for caspase 3 in vivo and lacks a cleavage site in the interaction domain.

AKAP95 plays a role in apoptotic nuclear morphological changes. To investigate a possible role for AKAP95 in apoptotic execution, we examined the effects of AKAP95 overexpression in cells. However, we did not observe any differences between transfected and nontransfected cells (data not shown and see Fig. 5B). As an alternative means of determining whether AKAP95 plays a role in apoptosis, we used siRNA (15) to deplete AKAP95 protein in HepG2 cells (Fig. 2). At 4 days after transfection of siRNA for AKAP95, the level of AKAP95 was decreased significantly (to <15%), whereas the levels of α-tubulin and procaspase 3 were unaffected (Fig. 2A). Therefore, the same number of cells transfected with siRNA for AKAP95 were reseeded, and apoptosis was induced by treatment with anti-Fas antibody. HepG2 cells transfected with siRNA for AKAP95 exhibited delayed nuclear morphological changes compared to control cells (Fig. 2B), suggesting that AKAP95 plays a role(s) in the nuclear morphological changes of apoptotic cells.

Association of caspase 3 with AKAP95 in yeast. Recently, we proposed that active caspase 3 is translocated in association with a substrate-like protein(s) from the cytoplasm into the nucleus in apoptotic cells (22). A carrier protein for the nuclear translocation of active caspase 3 would not be expected to be a caspase substrate per se because a typical enzyme-substrate complex is not stable but rather should associate reasonably stably with caspase 3 and should have a functional NLS. AKAP95 has all of these properties and therefore is a candidate for a carrier protein of caspase 3. To investigate this possibility, we next defined the AKAP95-binding region in caspase 3 using direct yeast two-hybrid assays (Table 1). Cotransformation of pBTM-casp3-p12p17^m, which expresses both p17 containing C163S mutation and p12 subunits of caspase 3, and pGAD-AKAP95 (clone 13 6-687) yielded His$^+$ transformants that were also β-galactosidase positive. However, neither caspase 3-p12 nor caspase 3-p17^m alone was able to bind to AKAP95, indicating that both subunits of caspase 3 are required for its binding to AKAP95. Furthermore, the R207E mutation, which prevents recognition of the P3 amino acid of substrates by caspase 3 (32, 42) and inhibited the nuclear translocation of active caspase 3 (22), abolished the association with AKAP95. These results suggest that the interaction of caspase 3 and AKAP95 may be analogous to an enzyme-substrate interaction.

TABLE 1. Association of AKAP95 with active caspase 3 in yeast two-hybrid assays[a]

pGAD (Gal4 AD)[b]	pBTM (LexA BD)[c]	β-Galactosidase activity[d]
AKAP95 (#13 6-687)	casp3-p12p17^m	+
AKAP95 (#13 6-687)	casp3-p12^{m}p17^m	−
AKAP95 (#13 6-687)	casp3-p17^m	−
AKAP95 (#13 6-687)	casp3-p12	−
AKAP95 (#13 6-47)	casp3-p12p17^m	−
AKAP95 (#13 6-341)	casp3-p12p17^m	−
AKAP95 (#13 6-542)	casp3-p12p17^m	−
AKAP95 (#13 340-429)	casp3-p12p17^m	−
AKAP95 (#13 428-687)	casp3-p12p17^m	+
AKAP95 (#13 544-687)	casp3-p12p17^m	+
AKAP95 (#13 544-670)	casp3-p12p17^m	−
AKAP95 (#13 556-687)	casp3-p12p17^m	+
AKAP95 (#13 556-679)	casp3-p12p17^m	+
AKAP95 (#13 544-687 E675G)	casp3-p12p17^m	−
AKAP95 (#13 544-687 T677G)	casp3-p12p17^m	−

[a] Yeast L40 cells were cotransfected with expression plasmids for Gal4 activation domain (Gal4 AD) fusion proteins and for LexA DNA-binding domain (LexA BD) fusion proteins as indicated.

[b] "#13" represents the clone number that was originally identified by yeast two-hybrid screening (21). The numbers (6-687, etc.) correspond to the encoded amino acids of AKAP95.

[c] pBTM-casp3-p12p17^m was used for expression of caspase 3-p12 and caspase 3-p17(C163S), pBTM-casp3-p12^{m}p17^m was used for expression of caspase 3-p12(R207E) and caspase 3-p17(C163S), pBTM-casp3-p17^m was used for expression of caspase 3-p17(C163S), and pBTM-casp3-p12 was used for expression of caspase 3-p12.

[d] Each transformation mixture was plated on a synthetic dropout plate lacking leucine, tryptophan, and histidine. Filter assays for β-galactosidase activity were performed to detect interactions between fusion proteins. +, development of blue color within 2 h; −, no growth of transformed yeast colonies.

Next we defined the active caspase 3-binding region in AKAP95 using yeast two-hybrid assays (Table 1). Various deletion mutants of AKAP95 were fused to the Gal4 activation domain and transformed into yeast with pBTM-casp3-p12p17^{m}. Cotransformation of pGAD-AKAP95 (clone 13, 544-687) or pGAD-AKAP95 (clone 13, 556-679), but not pGAD-AKAP95 (clone 13, 544-670), with pBTM-casp3-p12p17^{m} conferred the His^{+} phenotype and β-galactosidase activity, indicating that the binding site of AKAP95 for active caspase 3 is present in the C-terminal region (amino acids 556 to 679) of AKAP95 and that amino acids 671 to 679 are required for association with active caspase 3 in yeast. Although a consensus caspase 3 cleavage sequence (DXXD) was not found in this region, the tetrapeptide ($E^{675}QTG^{678}$), which is related to the caspase 3 cleavage site in gelsolin (DQTD) (21), is present (see Fig. 5C). To test whether this sequence is essential for binding to active caspase 3, we constructed E675G and T677G point mutants in a C-terminal fragment of mouse AKAP95 (amino acids 544 to 687). These mutations abolished the interaction of AKAP95 with active caspase 3 in yeast, suggesting that the $E^{675}QTG^{678}$ sequence in AKAP95 is required for binding to active caspase 3, possibly because this sequence functions as a noncleavable pseudosubstrate site for caspase 3. The fact that nuclear translocation of active caspase 3 did not require Arg^{64} or Cys^{163} (22), both of which are essential for recognition of, and cleavage after, Asp at P1 position (32, 42), is consistent with the absence of Asp at the position corresponding to P1 in the EQTG active caspase 3 binding site of mouse AKAP95.

Association of caspase 3 with AKAP95 in vivo. Next we tested whether an in vivo association between active caspase 3 and AKAP95 could be detected by coimmunoprecipitation. GFP-tagged caspase 3 with or without mutations was transiently overexpressed in 293T cells together with human AKAP95. As a control for specificity, C-terminally GFP-tagged procaspase 7 was coexpressed with AKAP95. Even though the cells were not induced to undergo apoptosis, casp3-Wt-GFP, GFP-Δpro-casp3-Wt, and casp7-Wt-GFP were proteolytically activated, presumably as a result a stress, such as serum starvation or a toxic effect of liposomes during transfection, or the overexpression of the wild-type caspases (Fig. 3A, upper panel). After immunoprecipitation of AKAP95, coprecipitated GFP-fusion proteins were detected with anti-GFP antibody (Fig. 3A, middle panel). Proteolytically activated casp3-p12-GFP derived from casp3-Wt-GFP and GFP-casp3-p17 derived from GFP-Δpro-casp3-Wt, but not casp7-p12-GFP from casp7-Wt-GFP, were coprecipitated with AKAP95, suggesting that active caspase 3 interacts with AKAP95 in vivo and that the association of effector caspases with AKAP95 may be specific for caspase 3. These results are consistent with our findings that caspase 3, but not caspase 7, translocated from the cytoplasm to the nucleus in apoptotic cells (22). Interestingly, all of the unprocessed GFP-caspase fusion proteins were coprecipitated along with AKAP95, but the level of casp7-Wt-GFP precipitated was significantly less than that of casp3-Wt-GFP. Since the coprecipitation of N-terminally GFP-tagged caspase 3 with AKAP95 was less effective than that of C-terminally GFP tagged caspase 3, GFP fused to the N terminus of caspase 3-p17 may interfere with immunocomplex formation.

To further analyze the interaction between procaspase 3 and AKAP95 in vivo, immunoprecipitations were carried out from lysates of 293T cells that were transiently transfected with AKAP95 and procaspase 3 expression plasmids (Fig. 3B). Procaspase 3 and C-terminally HA- or DsRed-tagged pro-caspase 3 was coprecipitated with AKAP95, suggesting that procaspase 3 also interacts with AKAP95 in 293T cells.

We also tested whether coprecipitation of AKAP95 with caspase 3 could be detected. For this purpose, N-terminally His-tagged AKAP95 was transiently overexpressed in 293T cells together with C-terminally GFP-tagged procaspase 3, and GFP-tagged caspase 3 was immunoprecipitated with anti-GFP antibodies, followed by detection of coprecipitated His-tagged AKAP95 by immunoblotting with anti-Xpress monoclonal antibody. As shown in Fig. 3C, His-tagged AKAP95 was coprecipitated with GFP-tagged caspase 3, although we could not determine whether His-tagged AKAP95 was coprecipitated with GFP-tagged procaspase 3, caspase 3-p12, or both.

To determine whether association between endogenous AKAP95 and caspase 3 proteins could be detected, lysates from HepG2 cells treated with or without anti-Fas antibody were separated into supernatant and pellet fractions after lysis with digitonin and immunoprecipitated with anti-AKAP95 serum, followed by immunoblotting with anti-caspase 3 antibodies. When we used anti-caspase 3 polyclonal antibodies for immunoblotting, which recognize both procaspase 3 and caspase 3-p12 subunit (Fig. 1), no active caspase 3 coprecipitating with AKAP95 was detected (data not shown). However, we detected a low level (<1%) of procaspase 3 coprecipitating with AKAP95 in the supernatant fraction of normal cells by using anti-caspase 3 monoclonal antibody in immunoblotting (Fig. 3D), suggesting that AKAP95 interacts with procaspase 3 endogenously in the cytoplasm of normal cells.

Colocalization of active caspase 3 and AKAP95. If AKAP95 functions as a carrier protein to transport active caspase 3 from the cytoplasm into nucleus, AKAP95 is expected to localize to the cytoplasm in normal cells and colocalize with active caspase 3 in apoptotic nuclei. To test this, various human cell lines were fractionated into supernatant and pellet fractions after lysis with digitonin, using lamin B1 as a nuclear marker. As shown in Fig. 4A, procaspase 3 was present only in the supernatant fraction, and AKAP95 was present in both the pellet and the supernatant fractions from normal cells. Next, apoptotic HepG2 cells were fractionated into supernatant and pellet fractions (Fig. 4B, left panel). Although procaspase 3 was present in the supernatant fraction, the caspase 3-p17 subunit and AKAP95 were present in both the pellet and supernatant fractions. Furthermore, HepG2 cells were stained with anti-AKAP95 and anti-active caspase 3 antibodies after treatment with anti-Fas antibody or etoposide (Fig. 4B, right panel). Although AKAP95 was detected in both nuclei and cytoplasm, active caspase 3 was not detected in cells before induction of apoptosis. However, AKAP95 and active caspase 3 were both highly enriched in the region around condensed nuclei in apoptotic cells, indicating colocalization of active caspase 3 and AKAP95 in apoptotic cells.

Function of AKAP95 in apoptotic nuclear morphological changes. If AKAP95 functions to carry caspase 3 from the cytoplasm into the nucleus, overexpression of AKAP95 with NLS-inactivating mutations should inhibit nuclear translocation of

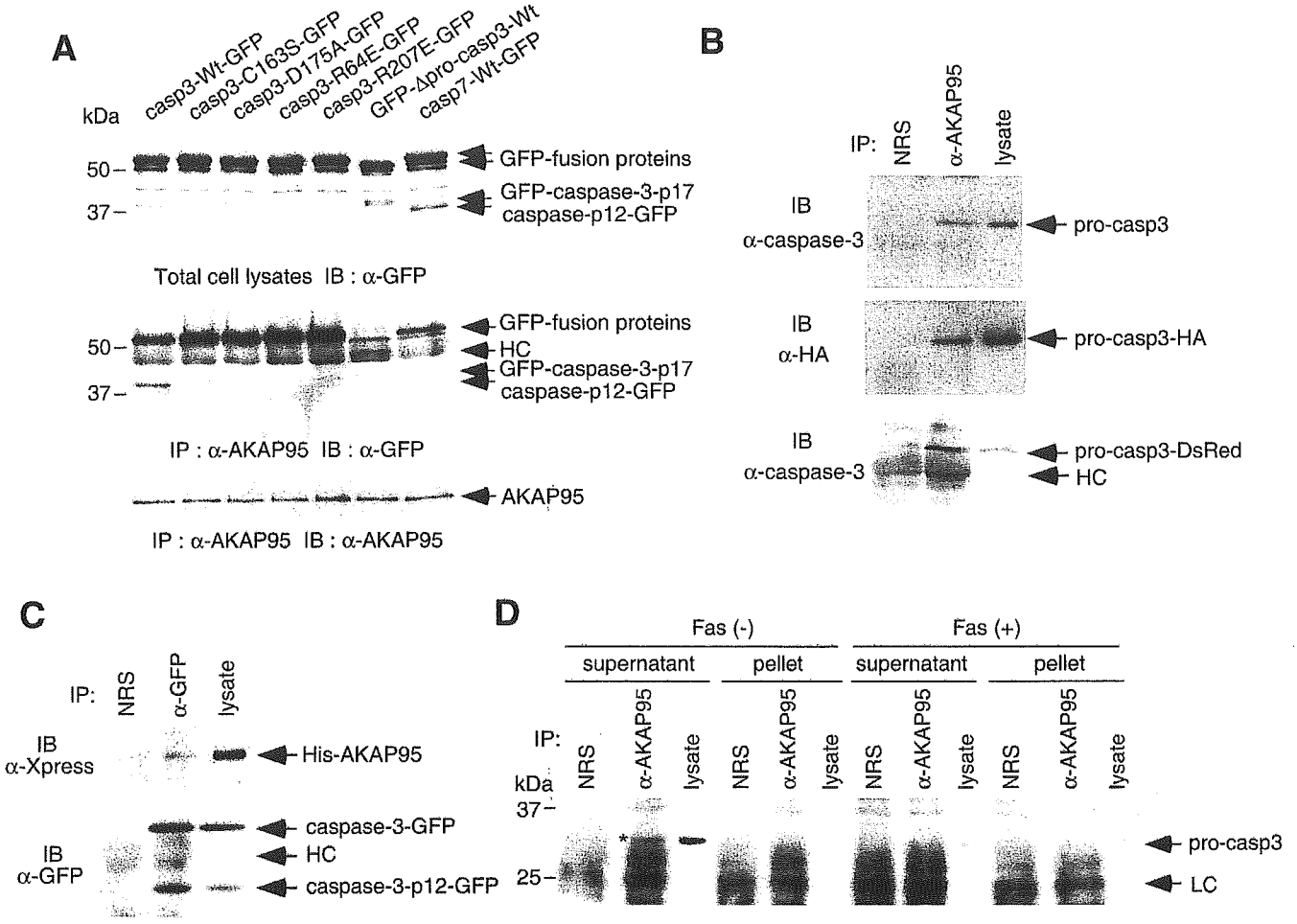

FIG. 3. In vivo association of caspase 3 with AKAP95. (A) Active caspase 3, but not active caspase-7, binds to AKAP95. 293T cells were transfected with caspase-GFP expression plasmids as indicated, together with pCAG-AKAP95-Wt. Casp3-C163S-GFP contained the mutation at the catalytic Cys, casp3-D175A-GFP contained the cleavage site mutation between the p17 and p12 subunits, casp3-R64E-GFP and casp3-R207E-GFP contained the mutations of substrate recognition sites, and GFP-Δpro-casp3-Wt was a prodomain deletion mutant. After incubation for 24 h, lysates were immunoprecipitated with anti-AKAP95 serum. The input lysates (upper panel) and the immunoprecipitates (middle and lower panels) were fractionated by SDS-PAGE and immunoblotted with anti-GFP monoclonal antibody (upper and middle panels) or anti-AKAP95 monoclonal antibody (lower panel). HC, heavy chain. (B) Coprecipitation of procaspase 3 with AKAP95 in transiently overexpressed 293T cells. Lysates from 293T cells transfected with either pCAG-casp3, pCAG-casp3-HA, or pcasp3-Wt-DsRed, together with pCAG-AKAP95-Wt, were immunoprecipitated with normal rabbit serum (NRS) or anti-AKAP95 serum (α-AKAP95). The immunoprecipitates and the input lysates were fractionated by SDS-PAGE and immunoblotted with anti-caspase 3 monoclonal antibody (upper and lower panels) or anti-HA monoclonal antibody (12CA5) (middle panel). (C) Coprecipitation of AKAP95 with caspase 3 in transiently overexpressed 293T cells. Lysates from 293T cells transfected pcDNA-AKAP95-Wt together with pcasp3-Wt-GFP were immunoprecipitated with normal rabbit serum (NRS) or anti-GFP polyclonal antibodies (α-GFP). The immunoprecipitates and the input lysates were fractionated by SDS-PAGE and immunoblotted with anti-Xpress monoclonal antibody that recognizes the leader peptide from the pcDNA3.1/His vector between His tag and AKAP95 (upper panel) or anti-GFP monoclonal antibody (lower panel). (D) Procaspase 3 binds to AKAP95 at endogenous protein levels. HepG2 cells treated with or without an agonistic anti-Fas antibody in the presence of actinomycin D for 12 h were divided into supernatant or pellet fractions after lysis with digitonin and immunoprecipitated as described in panel B. The immunoprecipitates and the input lysates were fractionated by SDS-PAGE and immunoblotted with anti-caspase 3 monoclonal antibody. The asterisk indicates the procaspase 3 coprecipitated with AKAP95. LC, light chain.

active caspase 3 by sequestration in the cytoplasm and thus prevent nuclear morphological changes in apoptotic cells. AKAP95 has potential overlapping bipartite NLSs (Fig. 5A) at amino acids 289 to 305 and amino acids 290 to 306. To identify amino acids necessary for the nuclear translocation of AKAP95, we constructed point mutants of basic amino acids in the putative NLS of human AKAP95 and fused these mutants to the C terminus of GFP. Mutation of Arg^{290} to Ser in AKAP95 (AKAP95-1M) had no effect on the nuclear localization of GFP-AKAP95 fusion protein (Fig. 5B), whereas a double mutation of Lys^{304} to Asn and Arg^{305} to Ser (AKAP95-2M) dramatically impaired nuclear accumulation of the fusion protein. Furthermore, combined mutation of R290S/K304N/R305S (AKAP95-3M) completely prevented nuclear localization of the AKAP95-GFP fusion protein (Fig. 5B). These results indicated that the overlapping bipartite NLSs located at amino acids 289 to 306 are essential for the nuclear import of AKAP95. Furthermore, introduction of the T681G point mutation (T677G in mouse AKAP95) (Table 1 and

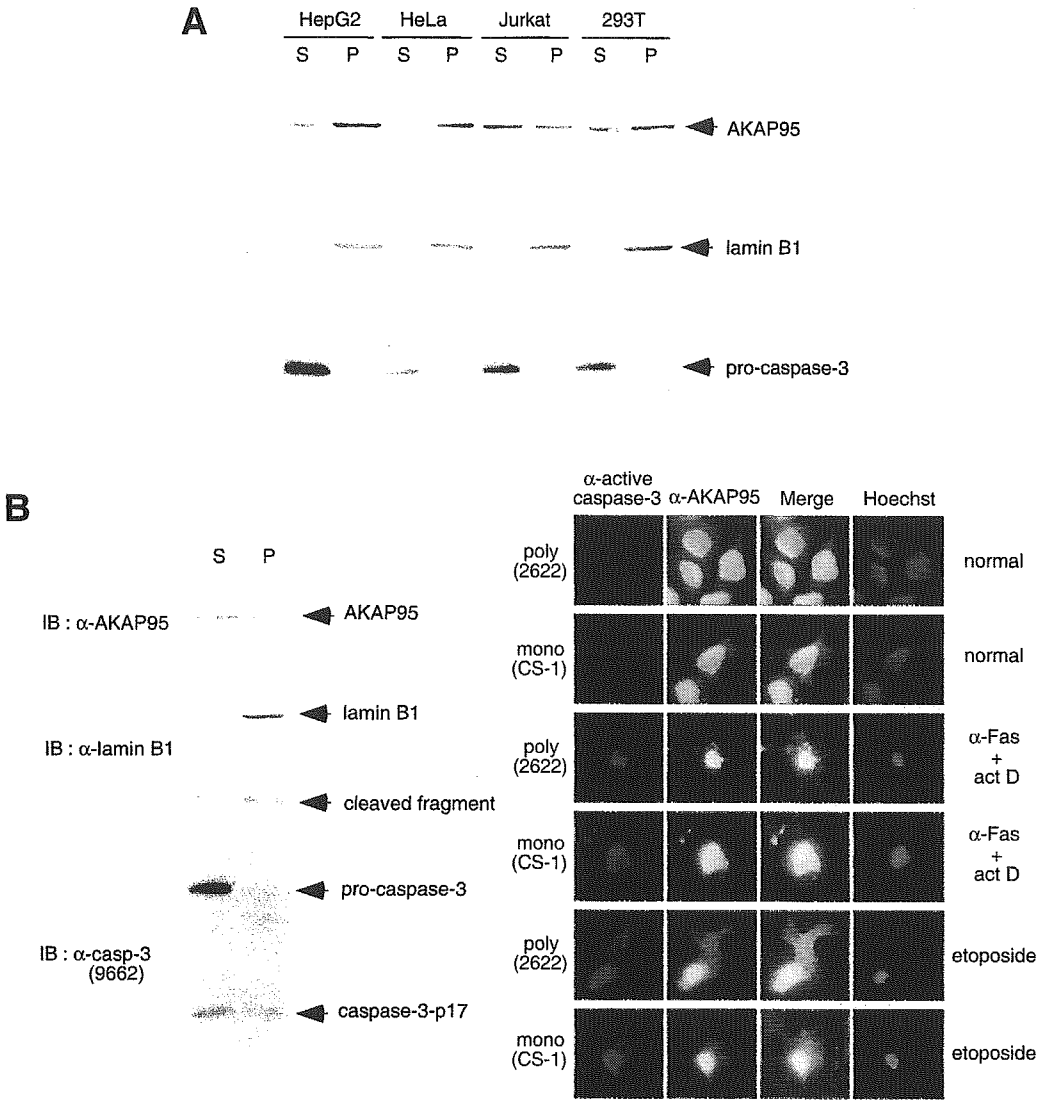

FIG. 4. Localization of caspase 3 and AKAP95. (A) Localization of AKAP95 and procaspase 3 in normal cells. HepG2, HeLa, Jurkat, and 293T cells were harvested and fractionated into pellet (P) and supernatant (S) fractions after lysis with digitonin. Each fraction was subjected to SDS-PAGE and immunoblotted with anti-AKAP95 monoclonal antibody (upper panel), anti-lamin B1 polyclonal antibodies as a nuclear fraction marker (middle panel), or anti-caspase 3 monoclonal antibody (lower panel). (B) Colocalization of active caspase 3 and AKAP95 in apoptotic cells. HepG2 cells treated with an agonistic anti-Fas antibody in the presence of actinomycin D for 12 h were fractionated as described for panel A. Each fraction was subjected to SDS-PAGE and immunoblotted with anti-AKAP95 monoclonal antibody, anti-lamin B1 polyclonal antibodies, or anti-caspase 3 polyclonal antibodies (antibody 9662; Cell Signaling Technology) that detect both procaspase 3 and caspase 3-p17 as indicated (left panel). HepG2 cells were treated without or with an agonistic anti-Fas antibody in the presence of actinomycin D for 12 h or with etoposide for 40 h. After fixation and permeabilization, the cells were incubated with anti-active caspase 3 polyclonal (antibody 2622) or monoclonal (CS-1) antibodies, with anti-AKAP95 monoclonal or polyclonal antibodies, and with Texas Red (TXRD)- or fluorescein isothiocyanate-labeled secondary antibodies, followed by staining the nuclei with Hoechst 33342 (right panel).

Fig. 5C) into AKAP95-Wt and AKAP95-3M did not affect the localization of GFP-AKAP95-Wt and GFP-AKAP95-3M, respectively (Fig. 5B).

To test whether overexpression of AKAP95 with NLS mutations inhibits nuclear morphological changes in apoptotic cells, GFP-AKAP95 with or without NLS mutations was transiently expressed in HepG2 cells, which were subsequently treated with anti-Fas antibody. GFP-AKAP95-2M and -3M had the greatest ability to prevent apoptotic nuclear morphological changes (Fig. 5D). Similar effects were also observed in HeLa cells (data not shown). Expression of GFP-AKAP95-Wt-T681G, which should not bind active caspase 3, did not decrease the percentage of cells with normal nuclear morphology compared to GFP-AKAP95-Wt (Fig. 5E), indicating that AKAP95 does not simply function as a competitive inhibitor of active caspase 3. Moreover, the T681G mutation partially impaired the protective effect of GFP-AKAP95-3M on apoptotic nuclear morphological changes in HepG2 cells (Fig. 5E). We could not determine whether this effect is a result of its inability to bind to active caspase 3 because the expression level of GFP-AKAP95-3M-T681G was lower than that of GFP-AKAP95-3M (<30% [data not shown]). However, this result

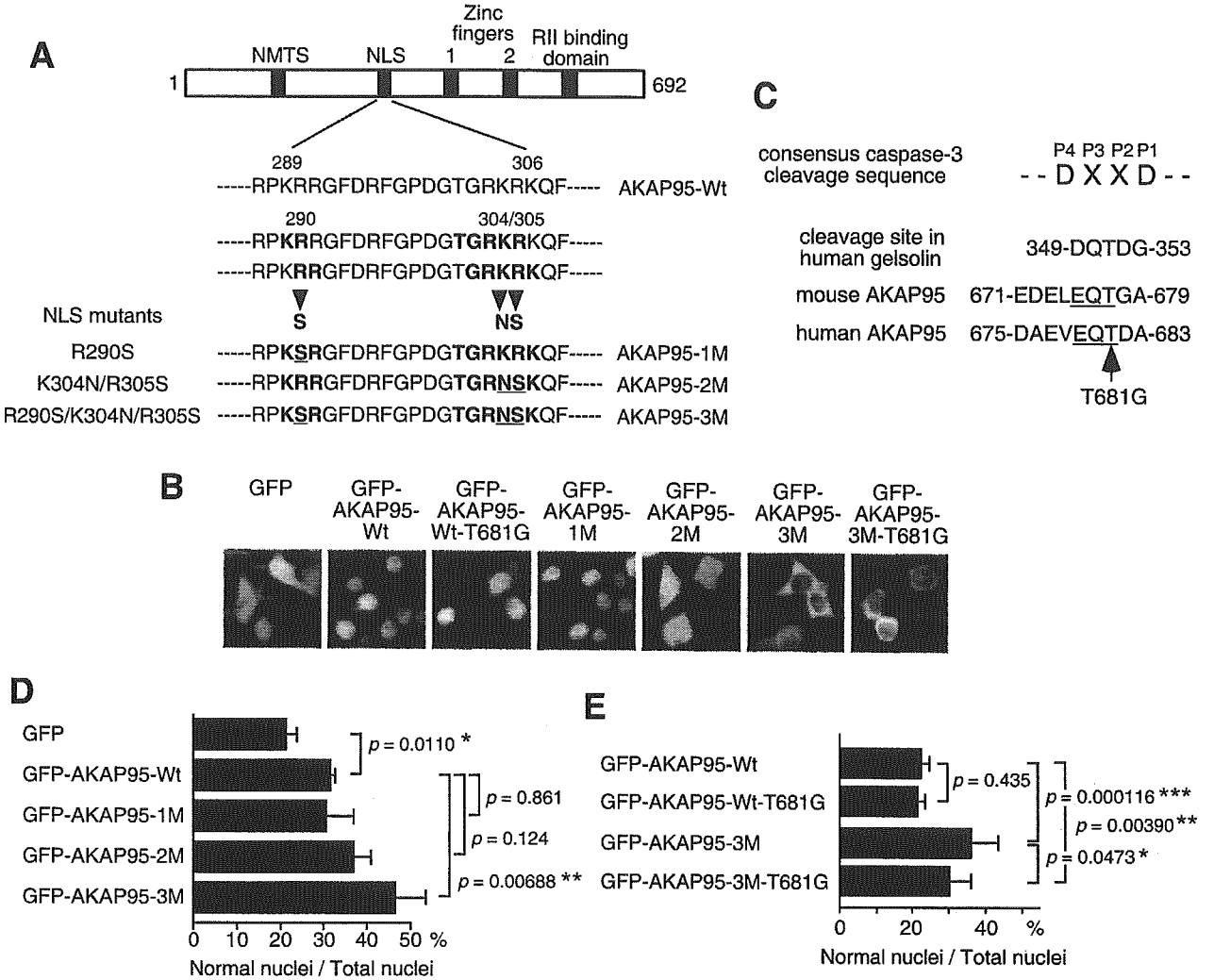

FIG. 5. Function of AKAP95 in nuclear morphological changes of apoptotic cells. (A) Diagram showing the NLS mutations of AKAP95. Mutated amino acid residues are underlined. NMTS, nuclear matrix targeting site; RII, type II PKA regulatory subunit. (B) Localization of GFP-AKAP95 fusion proteins with or without mutations. 293T cells were transiently transfected with GFP or GFP-AKAP95 expression plasmids as indicated. After 18 h, cells were observed by fluorescence microscopy. (C) Amino acid sequences of active caspase 3 binding region in AKAP95. Conserved EQT sequence between human and mouse AKAP95 is underlined. (D and E) Overexpression of AKAP95 with NLS mutations inhibits nuclear morphological changes. HepG2 cells were transfected with GFP-AKAP95 expression plasmids as indicated. After 24 h, cells were treated with an agonistic anti-Fas antibody in the presence of actinomycin D for 12 h (D) or 15 h (E) and collected and fixed with 3.7% formaldehyde for 10 min. After staining with Hoechst 33342, the percentage of the cells showing normal nuclei to the total transfected cells was measured. At least 100 GFP-positive cells were counted for each measurement in all experiments. The data (mean ± the standard deviation) were obtained from at least three independent experiments. Significant test results (P values) are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

clearly indicates that the protective effect of GFP-AKAP95-3M on apoptotic nuclear morphological changes depends on the expression of this protein. Collectively, our results showed that AKAP95 is a possible candidate of carrier proteins for the nuclear translocation of active caspase 3.

DISCUSSION

Mechanisms of nuclear translocation of active caspase 3. We have identified here AKAP95 as a caspase 3-binding protein that functions as a potential cytoplasm to nucleus carrier protein for caspase 3 in the process of apoptosis execution.

Although we detected an interaction of procaspase 3 with AKAP95 in normal cells, we did not observe nuclear accumulation of procaspase 3, despite the fact that most AKAP95 was nuclear. However, since the population of procaspase 3 that binds to AKAP95 is very small, nuclearly localized procaspase 3 may be hard to detect, even if procaspase 3 is imported into nuclei in association with AKAP95. Alternatively, it is possible that procaspase 3 binding to AKAP95 prevents nuclear translocation of the bound AKAP95 by masking the AKAP95 NLS. The binding of procaspase 3 to AKAP95 may also play a role in placing procaspase 3 close to AKAP95, thereby allowing caspase 3 to have easy access to the binding site in the C-terminal region of AKAP95 once the associated caspase 3 molecule is activated.

We detected an association of endogenous procaspase 3 with AKAP95 in normal cells, but we were unable to detect an

interaction of active caspase 3 with AKAP95 at endogenous protein levels. The failure to detect binding of active caspase 3 to AKAP95 at endogenous protein levels may be explained by a relatively small population of apoptotic cells, in which active caspase 3 binds to AKAP95, because apoptotic execution proceeds so fast (19, 31, 38, 41), or because the association between AKAP95 and activated caspase 3 is a weak and transient one, making it difficult to detect, especially because of the low sensitivity of anti-caspase 3 polyclonal antibodies for immunoblotting. In addition, degradation of active caspase 3 by the ubiquitin-proteasome pathway in apoptotic cells at early times reduces the amount of active caspase 3 (6, 20, 37).

Unexpectedly, we detected a weak interaction of procaspase 7 with AKAP95 by coimmunoprecipitation, but neither procaspase 7 nor activated caspase 7 was found to accumulate in nuclei (22). Although the prodomains of procaspase 3 and procaspase 7 are not conserved, caspase 3 and caspase 7 are highly conserved (54% identity) and have similar substrate specificities (40), and therefore procaspase 7 might be able to bind to the region containing the active caspase 3 binding site of AKAP95. Determination of the precise binding site of AKAP95 to procaspase 3 as well as procaspase 7 will be needed to resolve this issue.

The caspase substrate-binding groove is shaped by four surrounding loops, L1, L2, L3, and L4, whose sequences are highly conserved between caspase 3 and caspase 7 (35). However, the L2′ loop sequences, which correspond to the N-terminal region of the small subunits and are essential for substrate recognition (4, 5), are not conserved between caspase 3 and caspase 7, and this may explain why active caspase 3, but not active caspase 7, bound to AKAP95 and why only active caspase 3 accumulated in the nucleus.

From the results presented here, we can envision a molecular mechanism for the nuclear translocation of active caspase 3. In normal cells, a fraction of procaspase 3 molecules binds to AKAP95 in the cytoplasm. In response to apoptotic signals, procaspase 3 that is not bound to AKAP95 is activated and then cleaves cytoplasmic substrates, leading to apoptotic cytoplasmic changes. Procaspase 3 bound to AKAP95 is also activated, and the activated caspase 3 can remain bound to the C-terminal region of AKAP95. Since the population of procaspase 3 bound to AKAP95 in normal cells was very low, activated caspase 3 generated from procaspase 3 that was not bound to AKAP95 might also bind to AKAP95 after activation and be translocated into nucleus where it then cleaves nuclear substrates, leading to apoptotic nuclear morphological changes. Since neither the overexpression of AKAP95 NLS mutants nor siRNA-mediated depletion of AKAP95 completely abolished active caspase 3 nuclear entry, other active caspase 3 carriers might exist. Alternatively, the residual levels of AKAP95 might be sufficient for translocation of a small pool of active caspase 3 into the nucleus, where it acts on the nuclear pore from the inside and thereby allows larger proteins to diffuse in. It is reported that caspase-dependent disassembly of nuclear pores and disruption of the nucleocytoplasmic barrier precede nuclear entry of caspase 3 and DNA fragmentation mediated by caspase 3-dependent cleavage of ICAD/DFF45 (17, 23, 24), suggesting that dismantling of nuclear pores is essential for the early step of apoptotic nuclear changes. The nuclear pore membrane protein POM121, which is believed to play essential roles in formation of nuclear pores by anchoring other nucleoporins to the nuclear membrane, is cleaved in a caspase 3-dependent manner before nucleosomal DNA degradation during apoptosis (23, 24). Therefore, it seems possible that POM121 is a potential substrate for caspase 3 in nuclear pores at the early step of apoptotic nuclear morphological changes.

Regulation of nuclear morphological changes in apoptotic cells. Caspase 3 plays essential roles in apoptotic execution, especially in the nuclear changes in apoptotic cells, as demonstrated by studies of caspase 3 knockout cells (43, 44). Although caspase-activated DNase (CAD)/DNA fragmentation factor (DFF) 40 and apoptotic chromatin condensation inducer in the nucleus (Acinus) were identified in the cytoplasmic fraction of apoptotic cells (16, 27, 33), CAD/DFF40 and Acinus are suggested to be localized in the nuclei even before apoptosis induction (26, 27, 33). Furthermore, many nuclear substrates for caspase 3 have been identified (11, 12, 18, 39). Recently, Cheung et al. (7) reported that apoptotic chromatin condensation is dependent on phosphorylation of histone H2B that is mediated by caspase 3-cleaved Mst1 kinase. Therefore, nuclearly translocated active caspase 3 may cleave and activate nuclearly localized caspase substrates such as DFF, Acinus, lamins, or Mst1 kinase, leading to apoptotic nuclear morphological changes. In addition, AKAP95 plays essential roles in mitotic chromatin condensation by recruiting PKA and the condensin complex onto chromatin (9, 13, 36). Therefore, these components might be substrates for caspase 3 during apoptotic execution and play a role in apoptotic chromatin condensation.

ACKNOWLEDGMENT

We thank Joel D. Leverson and Han-Kuei Huang for critical reading of the manuscript, Vishva M. Dixit for the pcDNA3/Yama plasmid, Kjetil Tasken for the human AKAP95 cDNA, John D. Scott for the anti-AKAP95 serum, and Jun-ichi Miyazaki for the pUC-CAGGS plasmid.

This study was supported in part by Grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan (to S.K.) and by Public Health Service grants CA82683 and CA14195 from the National Cancer Institute (to T.H.). T.H. is a Frank and Else Schilling American Cancer Society Professor.

REFERENCES

1. **Akileswaran, L., J. W. Taraska, J. A. Sayer, J. M. Gettemy, and V. M. Coghlan.** 2001. A-kinase-anchoring protein AKAP95 is targeted to the nuclear matrix and associates with p68 RNA helicase. J. Biol. Chem. **276:** 17448–17454.
2. **Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan.** 1996. Human ICE/CED-3 protease nomenclature. Cell **87:**171.
3. **Baird, G. S., D. A. Zacharias, and R. Y. Tsien.** 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. USA **97:**11984–11989.
4. **Chai, J., E. Shiozaki, S. M. Srinivasula, Q. Wu, P. Dataa, E. S. Alnemri, and Y. Shi.** 2001. Structural basis of caspase-7 inhibition by XIAP. Cell **104:** 769–780.
5. **Chai, J., Q. Wu, E. Shiozaki, S. M. Srinivasula, E. S. Alnemri, and Y. Shi.** 2001. Crystal structure of a procaspase-7 zymogen: mechanisms of activation and substrate binding. Cell **107:**399–407.
6. **Chen, L., L. Smith, Z. Wang, and J. B. Smith.** 2003. Preservation of caspase 3 subunits from degradation contributes to apoptosis evoked by lactacystin: any single lysine or lysine pair of the small subunit is sufficient for ubiquitination. Mol. Pharmacol. **64:**334–345.
7. **Cheung, W. L., K. Ajiro, K. Samejima, M. Kloc, P. Cheung, C. A. Mizzen, A. Beeser, L. D. Etkin, J. Chernoff, W. C. Earnshaw, and C. D. Allis.** 2003. Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. Cell **113:**507–517.

8. **Coghlan, V. M., L. K. Langeberg, A. Fernandez, N. J. Lamb, and J. D. Scott.** 1994. Cloning and characterization of AKAP 95, a nuclear protein that associates with the regulatory subunit of type II cAMP-dependent protein kinase. J. Biol. Chem. **269:**7658–7665.
9. **Collas, P., K. Le Guellec, and K. Tasken.** 1999. The A-kinase-anchoring protein AKAP95 is a multivalent protein with a key role in chromatin condensation at mitosis. J. Cell Biol. **147:**1167–1180.
10. **Colledge, M., and J. D. Scott.** 1999. AKAPs: from structure to function. Trends Cell Biol. **9:**216–221.
11. **Cryns, V., and J. Yuan.** 1998. Proteases to die for. Genes Dev. **12:**1551–1570.
12. **Earnshaw, W. C., L. M. Martins, and S. H. Kaufmann.** 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. **68:**383–424.
13. **Eide, T., C. Carlson, K. A. Tasken, T. Hirano, K. Tasken, and P. Collas.** 2002. Distinct but overlapping domains of AKAP95 are implicated in chromosome condensation and condensin targeting. EMBO Rep. **3:**426–432.
14. **Eide, T., V. Coghlan, S. Orstavik, C. Holsve, R. Solberg, B. S. Skalhegg, N. J. Lamb, L. Langeberg, A. Fernandez, J. D. Scott, T. Jahnsen, and K. Tasken.** 1998. Molecular cloning, chromosomal localization, and cell cycle-dependent subcellular distribution of the A-kinase anchoring protein, AKAP95. Exp. Cell Res. **238:**305–316.
15. **Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411:**494–498.
16. **Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata.** 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature **391:**43–50.
17. **Faleiro, L., and Y. Lazebnik.** 2000. Caspases disrupt the nuclear-cytoplasmic barrier. J. Cell Biol. **151:**951–959.
18. **Fischer, U., R. U. Janicke, and K. Schulze-Osthoff.** 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. **10:**76–100.
19. **Goldstein, J. C., N. J. Waterhouse, P. Juin, G. I. Evan, and D. R. Green.** 2000. The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. Nat. Cell Biol. **2:**156–162.
20. **Huang, H., C. A. Joazeiro, E. Bonfoco, S. Kamada, J. D. Leverson, and T. Hunter.** 2000. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. J. Biol. Chem. **275:**26661–26664.
21. **Kamada, S., H. Kusano, H. Fujita, M. Ohtsu, R. C. Koya, N. Kuzumaki, and Y. Tsujimoto.** 1998. A cloning method for caspase substrates that uses the yeast two-hybrid system: cloning of the antiapoptotic gene gelsolin. Proc. Natl. Acad. Sci. USA **95:**8532–8537.
22. **Kamada, S., U. Kikkawa, Y. Tsujimoto, and T. Hunter.** 2005. Nuclear translocation of caspase 3 is dependent on its proteolytic activation and recognition of a substrate-like protein(s). J. Biol. Chem. **280:**857–860.
23. **Kihlmark, M., C. Rustum, C. Eriksson, M. Beckman, K. Iverfeldt, and E. Hallberg.** 2004. Correlation between nucleocytoplasmic transport and caspase-3-dependent dismantling of nuclear pores during apoptosis. Exp. Cell Res. **293:**346–356.
24. **Kihlmark, M., G. Imreh, and E. Hallberg.** 2001. Sequential degradation of proteins from the nuclear envelope during apoptosis. J. Cell Sci. **114:**3643–3653.
25. **Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic, and R. A. Flavell.** 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature **384:**368–372.
26. **Lechardeur, D., L. Drzymala, M. Sharma, D. Zylka, R. Kinach, J. Pacia, C. Hicks, N. Usmani, J. M. Rommens, and G. L. Lukacs.** 2000. Determinants of the nuclear localization of the heterodimeric DNA fragmentation factor (ICAD/CAD). J. Cell Biol. **150:**321–334.
27. **Liu, X., P. Li, P. Widlak, H. Zou, X. Luo, W. T. Garrard, and X. Wang.** 1998. The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc. Natl. Acad. Sci. USA **95:**8461–8466.
28. **Mancini, M., D. W. Nicholson, S. Roy, N. A. Thornberry, E. P. Peterson, L. A. Casciola-Rosen, and A. Rosen.** 1998. The caspase 3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J. Cell Biol. **140:**1485–1495.
29. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
30. **Ohtsubo, T., S. Kamada, T. Mikami, H. Murakami, and Y. Tsujimoto.** 1999. Identification of NRF2, a member of the NF-E2 family of transcription factors, as a substrate for caspase-3(-like) proteases. Cell Death Differ. **6:**865–872.
31. **Rehm, M., H. DuBmann, R. U. Janicke, J. M. Tavare, D. Kogel, J. H. M. Prehn.** 2002. Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. J. Biol. Chem. **277:**24506–24514.
32. **Rotonda, J., D. W. Nicholson, K. M. Fazil, M. Gallant, Y. Gareau, M. Labelle, E. P. Peterson, D. M. Rasper, R. Ruel, J. P. Vaillancourt, N. A. Thornberry, and J. W. Becker.** 1996. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat. Struct. Biol. **3:**619–625.
33. **Sahara, S., M. Aoto, Y. Eguchi, N. Imamoto, Y. Yoneda, and Y. Tsujimoto.** 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature **401:**168–173.
34. **Samali, A., B. Zhivotovsky, D. P. Jones, and S. Orrenius.** 1998. Detection of pro-caspase 3 in cytosol and mitochondria of various tissues. FEBS Lett. **431:**167–169.
35. **Shi, Y.** 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell **9:**459–470.
36. **Steen, R. L., F. Cubizolles, K. Le Guellec, and P. Collas.** 2000. A kinase-anchoring protein (AKAP)95 recruits human chromosome-associated protein (hCAP)-D2/Eg7 for chromosome condensation in mitotic extract. J. Cell Biol. **149:**531–536.
37. **Suzuki, Y., Y. Nakabayashi, and R. Takahashi.** 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase 3 and enhances its antiapoptotic effect in Fas-induced cell death. Proc. Natl. Acad. Sci. USA **98:**8662–8667.
38. **Takemoto, K., T. Nagai, A. Miyawaki, and M. Miura.** 2003. Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. J. Cell Biol. **160:**235–243.
39. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science **281:**1312–1316.
40. **Thornberry, N. A., T. A. Rano, E. P. Peterson, D. M. Rasper, T. Timkey, M. Garcia-Calvo, V. M. Houtzager, P. A. Nordstrom, S. Roy, J. P. Vaillancourt, K. T. Chapman, and D. W. Nicholson.** 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. J. Biol. Chem. **272:**17907–17911.
41. **Tyas, L., V. A. Brophy, A. Pope, A. J. Rivett, and J. M. Tavare.** 2000. Rapid caspase 3 activation during apoptosis revealed using fluorescence-resonance energy transfer. EMBO Rep. **1:**266–270.
42. **Wei, Y., T. Fox, S. P. Chambers, J. Sintchak, J. T. Coll, J. M. C. Golec, L. Swenson, K. P. Wilson, and P. S. Charifson.** 2000. The structure of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. Chem. Biol. **7:**423–432.
43. **Woo, M., R. Hakem, M. S. Soengas, G. S. Duncan, A. Shahinian, D. Kagi, A. Hakem, M. McCurrach, W. Khoo, S. A. Kaufman, G. Senaldi, T. Howard, S. W. Lowe, and T. W. Mak.** 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. **12:**806–819.
44. **Zheng, T. S., S. F. Schlosser, T. Dao, R. Hingorani, I. N. Crispe, J. L. Boyer, and R. A. Flavell.** 1998. Caspase 3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis in vivo. Proc. Natl. Acad. Sci. USA **95:**13618–13623.

Cell Death and Differentiation (2005) 12, 1528–1534

www.nature.com/cdd

Review

Another way to die: autophagic programmed cell death

Y Tsujimoto*,[1,2] and S Shimizu[1,2]

[1] Laboratory of Molecular Genetics, Department of Medical Genetics, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[2] SORST of Japan Science Technology Corp., 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
* Corresponding author: Y Tsujimoto, Laboratory of Molecular Genetics, Department of Medical Genetics, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871 Japan. Tel: +81-6-6879-3360; Fax: +81-6-6879-3369; E-mail: tsujimot@gene.med.osaka-u.ac.jp

Received 06.5.05; revised 18.8.05; accepted 18.8.05
Edited by G Melino

Abstract

Programmed cell death (PCD) is one of the important terminal paths for the cells of metazoans, and is involved in a variety of biological events that include morphogenesis, maintenance of tissue homeostasis, and elimination of harmful cells. Dysfunction of PCD leads to various diseases in humans, including cancer and several degenerative diseases. Apoptosis is not the only form of PCD. Recent studies have provided evidence that there is another mechanism of PCD, which is associated with the appearance of autophagosomes and depends on autophagy proteins. This form of cell death most likely corresponds to a process that has been morphologically defined as autophagic PCD. The present review summarizes recent experimental evidence about autophagic PCD and discusses some aspects of this form of cell death, including the mechanisms that may distinguish autophagic death from the process of autophagy involved in cell survival.

Cell Death and Differentiation (2005) **12,** 1528–1534.
doi:10.1038/sj.cdd.4401777

Keywords: cell death; autophagy; apoptosis; Bcl-2; Bax

Abbreviations: PCD, programmed cell death; MEFs, mouse embryonic fibroblasts; 3-MA, 3-methyladenine; WT, wild-type; Glud2, glutamate receptor $\delta 2$

A Constantly Raised Concern for Autophagic Death

Apoptosis, a form of programmed cell death (PCD), has attracted considerable attention over the last 15 years and a significant part of the molecular mechanisms involved has already been unveiled. However, there is considerable evidence to suggest that PCD is not confined to apoptosis and that other mechanisms may also operate. One of these mechanisms is the so-called 'autophagic PCD',[1] which is a process that is associated with autophagosomes and autolysosomes.

Autophagy

Here, we briefly summarize the well-established process of autophagosome formation. Readers who are interested in more detailed information on autophagy should refer to several excellent recent reviews.[2–4]

Autophagosomes are double-membrane cytoplasmic vesicles that are designed to engulf various cellular constituents, including cytoplasmic organelles (Figure 1). Autophagosomes fuse to lysosomes to become autolysosomes, where sequestered cellular components are digested. Autophagosomes and autolysosomes are formed during a process called macroautophagy (hereafter this is referred to as 'autophagy'), which is activated by starvation conditions associated with deficiency of nutrients such as amino acids. Autophagy is also known to be activated by hypoxic conditions and high temperatures. Autophagy is a process by which cells undergo partial autodigestion (the term is derived from ancient Greek meaning to 'eat oneself') that prolongs survival for a short time under starvation conditions. It provides nutrients that are necessary to maintain cell viability. It has more recently been shown that autophagy is also involved in the killing of bacteria that are ingested by cells.[5,6]

The molecular basis of autophagy has been extensively studied, mainly in yeasts, by investigation of autophagy-defective mutants to identify the responsible genes (designated as *atg*). It is currently known that the basic mechanism of autophagy has been well conserved during evolution from the fact that diverse organisms, including yeasts, flies, and mammals, all carry a similar set of *atg* genes, although there are some significant differences between yeasts and higher eukaryotes. Autophagy is regulated by PI3 kinase type I and III. PI3 kinase type I is activated by growth factors like insulin and inhibits autophagy through PDK1 and AKT, which regulates mammalian target of rapamycin (mTOR). It is still unclear how activation of mTOR inhibits autophagy, but the mechanism may involve phosphorylation of Atg13, which is part of a protein complex with Atg1, a serine/threonine kinase. Since mTOR is regulated by many other proteins, regulation of the process of autophagy is likely to be very complex. PI3 kinase type III, which includes Atg6 in its complex, promotes the nucleation of autophagic vesicles (Figure 1). Expansion of autophagic vesicles is mediated by two ubiquitin-like conjugation systems: (1) the Atg12 pathway (involving Atg12 (ubiquitin-like), Atg7 (E1-like), Atg10 (E2-like), and Atg5) and (2) the Atg8 pathway (involving Atg8 (ubiquitin-like), Atg7 (E1-like), Atg3 (E2-like), and Atg4) (Figure 1). In the Atg12 pathway, Atg12 is conjugated to Atg5, while Atg8 is conjugated to phosphatidylethanolamine in the Atg8 pathway.

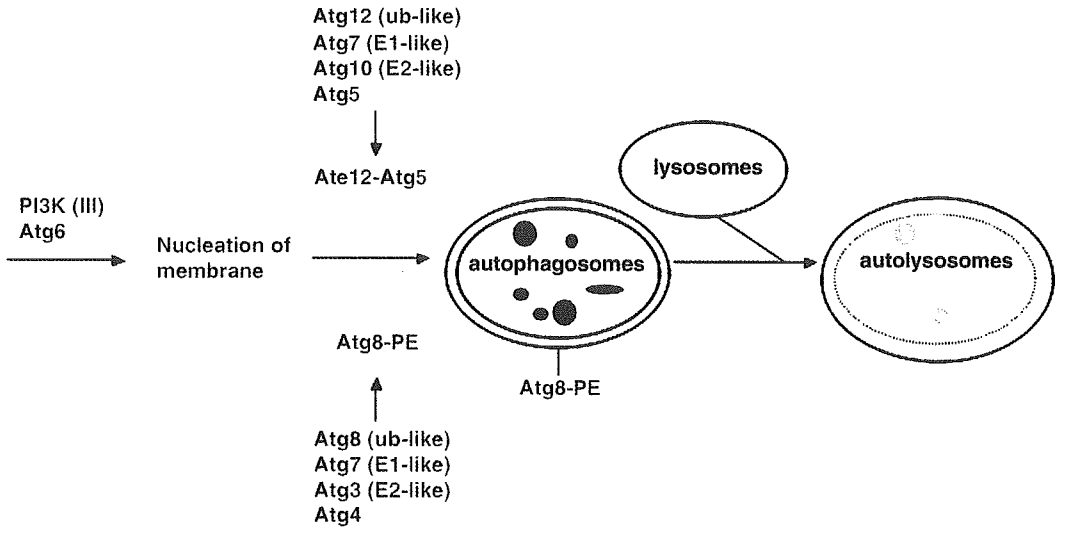

Figure 1 Diagram of the autophagic process. PI3 kinase type III and two ubiquitin-like conjugation systems are involved in the formation of autophagosomes. PE: phosphatidylethanolamine; ub: ubiquitin

Autophagic death

Autophagic cell death is mainly a morphologic definition (i.e. cell death associated with autophagosomes/autolysosomes), and there is still no conclusive evidence that a specific mechanism of autophagic death actually exists. However, it is quite conceivable that constitutive autophagy could eventually destroy a cell. This hypothesis as well as previous reports that cells with autophagic features often exist in regions where PCD is occurring seem to support the existence of autophagic cell death.[7] One question that constantly arises, however, is whether autophagic activity in dying cells is the cause of death or is actually an attempt to prevent it. Morphological and histochemical studies cannot prove a causative relationship between the autophagic process and cell death. In fact, there have recently been strong arguments that autophagic activity in dying cells might actually be a survival mechanism.

One reason why this issue has not been resolved is a lack of a suitable experimental system to investigate autophagic death. We recently found that cytotoxic stimuli activate autophagic death in cells that are protected against apoptosis, such as those expressing antiapoptotic Bcl-2 or Bcl-x_L, or those lacking both Bax and Bak (multidomain proapoptotic members of the Bcl-2 family that function as a gateway for a variety of apoptotic signals).[8] Lenardo's group has also found that cell lines such as L929 undergo autophagic death in the presence of z-VAD-fmk, a pancaspase inhibitor.[9]

Autophagic Programmed Death is an Alternative to Apoptosis

Bax/Bak-deficient mouse embryonic fibroblasts (MEFs) do not undergo apoptosis after exposure to a variety of apoptotic stimuli.[10,11] Instead, we have found that these cells die in a nonapoptotic manner when exposed to various cytotoxic agents, such as etoposide, staurosporine, and thapsigargin. MEFs exposed to these agents show (1) loss of clonogenicity (the ability to form colonies) and (2) positive staining with propidium iodide (a marker of plasma membrane disruption), findings which are compelling evidence of cell death when taken together.[8] Electron microscopy has revealed that drug-treated Bax/Bak-deficient cells contain numerous double-membrane vesicles[8] (also see Figure 2), which have been confirmed to be autophagosomes by the punctate distribution of GFP-LC3 (GFP-Atg8: a portion specifically concentrated on autophagosomes that is normally spread throughout the cytoplasm)[8] (also see Figure 3). This nonapoptotic form of cell death can be inhibited by suppressing autophagosome formation with autophagy inhibitors, such as 3-methyladenine (3-MA) and wortmannin, or by silencing Atg5 and Atg6, as assessed by several different methods.[8] Inhibition of cell death has been convincingly demonstrated by detection of improved clonogenicity[8] (also see Figure 4). Thus, these results indicate that autophagosome formation is required for cells to die after exposure to cytotoxic drugs, proving the existence of an alternative death mechanism to apoptosis that could be termed autophagic cell death. Independently, Lenardo's group has also shown by studies on several cell lines, including mouse L929 cells and human U937 cells, that

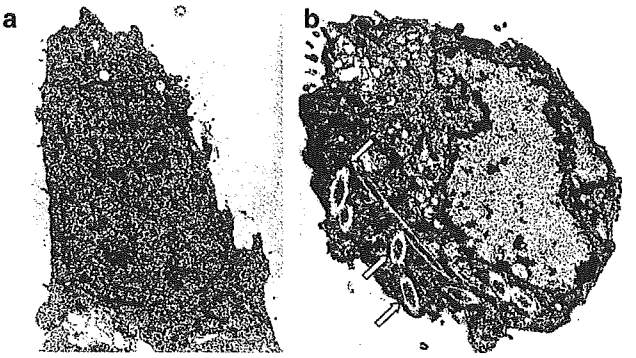

Figure 2 Electron micrograph of Bax/Bak-deficient MEFs with and without exposure to etoposide. (**a**) Control cell and (**b**) etoposide (20 μM)-treated cell. The etoposide-treated cell contains many autophagosomes (arrows)

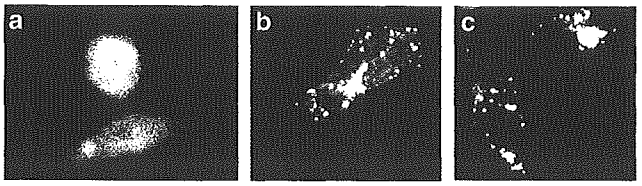

Figure 3 GFP-LC3 fluorescence in Bax/Bak-deficient MEFs. Bax/Bak-deficient MEFs were transfected with GFP-LC3 DNA and then cultured (**a**) without etoposide, (**b**) with etoposide (20 μM), and (**c**) in the absence of amino acids. Whereas untreated healthy cells show diffuse distribution of GFP-LC3, Bax/Bak-deficient MEFs exposed to etoposide or amino-acid depletion show a punctate distribution of GFP-LC3, indicating the formation of autophagosomes. LC3 is known to be concentrated on autophagosomes

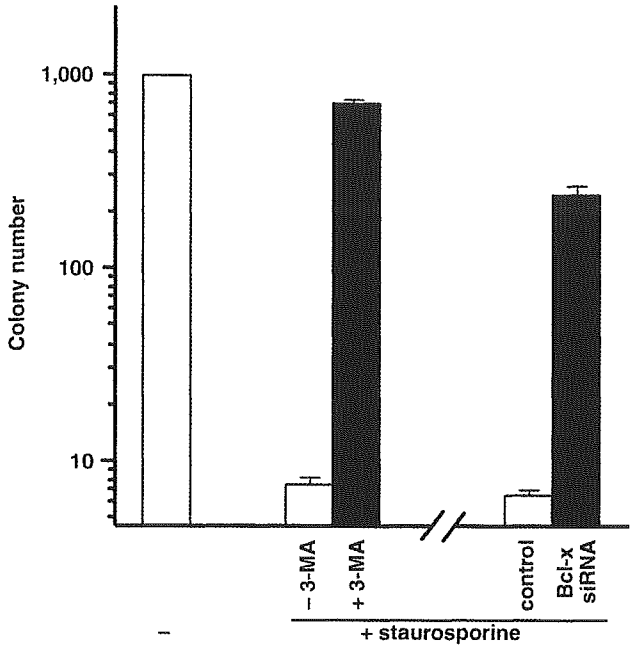

Figure 4 Inhibition of autophagic death in Bax/Bak-deficient MEFs by an autophagy inhibitor and by silencing of Bcl-x. Bax/Bak-deficient MEFs were left untreated (–) or treated (+) with 1 μM staurosporine for 24 h in the absence or presence of 10 mM 3-methyladenine (3-MA). Cells were also treated with and without Bcl-x siRNA and then with 1 μM staurosporine for 24 h. Cell viability was assessed by a clonogenicity assay: cells were washed and replated in regular medium to allow the formation of colonies

a pancaspase inhibitor induces nonapoptotic death associated with autophagic manifestations, which is inhibited by silencing of Atg5 and Atg7.[9]

Based on our observations and Lenardo's, we propose that a better definition of autophagic cell death would include an association with autophagosomes as well as dependence on autophagy proteins. In this regard, the death of Purkinje cells induced by the Lurcher mutation (a constitutive active mutation) of the δ2 glutamate receptor (Glud2) could be categorized as a type of autophagic death because it is associated with autophagosomes and is inhibited by 3-MA.[12] It has been shown that Glud2 interacts with nPIST (a PDZ protein specifically interacting with TC10) that binds to Beclin 1 (Atg6), suggesting a molecular link between Glud2 and the

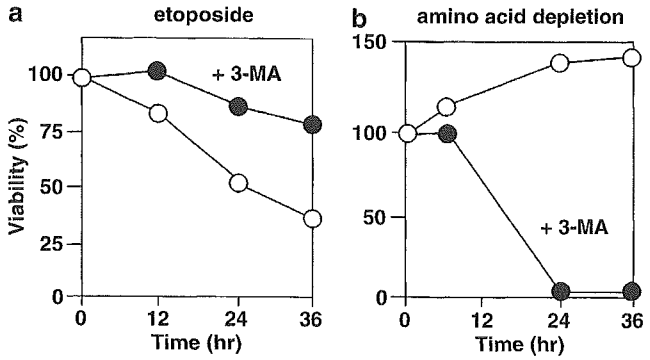

Figure 5 Inhibition of etoposide-induced death, but not amino-acid depletion-induced death, of Bax/Bak-deficient MEFs by 3-MA. Bax/Bak-deficient MEFs were incubated with 20 μM etoposide (**a**) or in the absence of amino acids (**b**) in the presence and absence of 10 mM 3-MA for the indicated times, and cell viability was assessed by the Cell Titer Blue assay[8]

autophagic machinery.[12] It was also recently reported that INFγ induces Atg5-dependent autophagic death.[13] On the other hand, the following situation should not be considered as autophagic death: when Bax/Bak-deficient MEFs are cultured in the absence of amino acids, the process of autophagy is activated to prolong survival (Figure 3c), but the cells eventually die of nutrient deficiency unless amino acids are provided. In this setting, inhibition of autophagy actually enhances cell death, indicating that such death does not depend on autophagy proteins (Figure 5). The same is true for the death of an IL3-dependent cell line induced by IL-3 deprivation, because autophagy is activated but does not itself cause death.[14] In these situations, autophagy is activated as a mechanism that prolong cell survival (Figure 6). We will discuss this issue further in the following sections.

Although it has been shown that Bax/Bak-deficient MEFs undergo autophagic death after exposure to etoposide, staurosporine, and thapsigargin,[8] neither X-ray irradiation nor TNF/cycloheximide induces autophagic death (our unpublished results), even though all of these stimuli induce apoptosis of wild-type (WT) MEFs. Similarly, in Bax/Bak-deficient thymocytes, autophagic death is readily induced by etoposide and staurosporine,[8] but not by dexamethasone (our unpublished results), although all of these reagents induce apoptosis in WT thymocytes. These results indicate that only certain death stimuli trigger the autophagic death of Bax/Bak-deficient cells, suggesting that particular signaling molecule(s) may be necessary for autophagic death, which is only activated by some cytotoxic stimuli.

It is also noteworthy that inhibition of caspases in WT MEFs treated with cytotoxic drugs inhibits apoptotic death but does not induce autophagic death.[8] On the other hand, overexpression of Bcl-2 or Bcl-x_L in WT MEFs (which is equivalent to Bax/Bak-deficiency in MEFs in terms of inhibiting apoptosis) blocks apoptosis but promotes autophagic death.[8] These results imply that induction of autophagic death is not merely a consequence of the inhibition of apoptosis, but rather is regulated by the Bcl-2 family of proteins. This observation taken together with the finding that inhibitors of mitochondrial functions, such as respiratory chain inhibitors, can block autophagic death (our unpublished

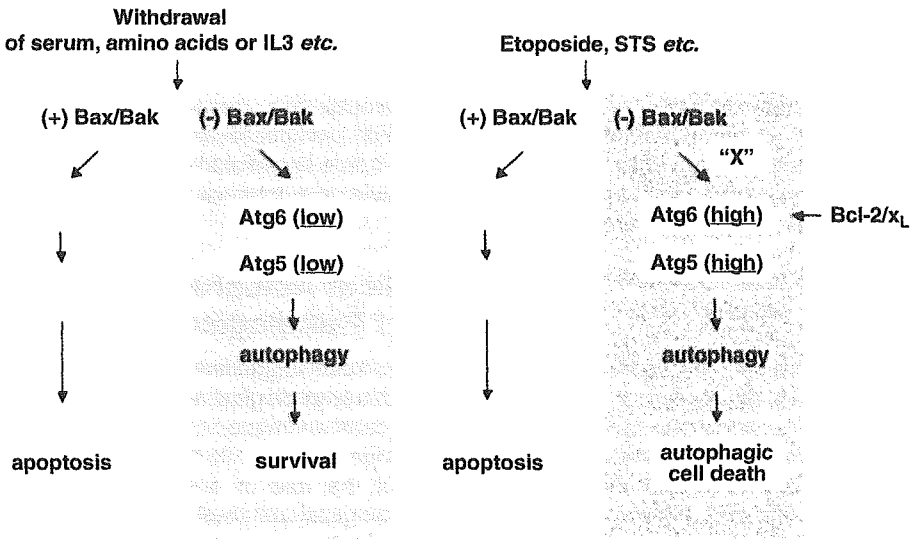

Figure 6 Autophagy and autophagic death. All stimuli (withdrawal of serum, amino acids, or lymphokines, and cytotoxic drugs such as etoposide and staurosporine) induce apoptosis of WT MEFs. When apoptosis is blocked by Bax/Bak-deficiency, only etoposide and staurosporine upregulate Atg5/Atg6 and activate autophagic death, whereas withdrawal of amino acids, serum, or lymphokines activate autophagy to prolong cell survival. Bcl-2/Bcl-x_L is required for Bax/Bak-deficient MEFs to undergo autophagic death and might enhance the autophagic process via interaction with Atg6. STS: staurosporine

results) suggests that the mitochondria need to be functioning to allow the induction of autophagic death at least in MEFs. This might be contrary to the situation in *Drosophila*, where autophagic activity is seen in dying cells of the salivary gland and mid guts along with activation of caspases.[15–17] Such findings imply that regulation of autophagic death varies considerably among different cells. In the fly, mitochondrial function might somehow be preserved even after caspase activation so that autophagic death occurs. Alternatively, autophagy may occur to more efficiently degrade the structural components of dying cells, and reduce the workload for phagocytes, and may not be a mechanism of cell death.

Studies performed with several culture systems have provided evidence that cells possess a novel death mechanism that depends on autophagy proteins.[8,9] However, it can be argued that these are all nonphysiological models, so these findings do not necessarily indicate the physiological occurrence of this form of cell death. Of course, there are a number of *in vivo* situations where apoptosis is blocked at the mitochondrial level, such as upregulation of Bcl-2 or Bcl-x_L. So, our findings imply that these cells may have the potential to undergo autophagic death. In any case, although the results should be treated with caution, these culture systems provide an opportunity to investigate the autophagic death in detail, and such studies provide new evidence about the physiological and pathological occurrence of the autophagic death *in vivo*.

Differentiating Autophagic Death from Autophagy

The same set of the proteins (Atg5, Atg6, and Atg7) and the formation of autophagosomes are involved in both autophagic cell death and autophagy that promotes cell survival.[8,9] Therefore, a question arises as to how these two opposite outcomes are regulated. Bax/Bak-deficient MEFs show activation of autophagy in response to both starvation and drug treatment, thus providing a useful tool to answer this question. One hint revealed by our recent study is a significant difference in the expression of Atg5 and Atg6 proteins during these two processes, since the levels of both proteins remain low during autophagy (survival), whereas these proteins are highly upregulated during autophagic death.[8] Thus, it seems that the same set of proteins is involved in both autophagic death and autophagy (survival), but their regulation is substantially different during each process (Figure 6). Certainly, it needs to be experimentally proven whether this difference in the expression of Atg5 and Atg6 proteins leads to the selection of autophagic death or autophagy that promotes cell survival.

Depletion of amino acids or serum from WT MEFs induces apoptosis, but if apoptosis is blocked (e.g. by Bax/Bak-deficiency), activation of autophagy provides the nutrients necessary for cells to survive. Therefore, blocking autophagy causes cell death to be enhanced (see Figures 5 and 6). On the other hand, drugs such as etoposide or staurosporine upregulate the expression of some Atg proteins, leading to Atg protein-dependent cell death (Figures 5 and 6). It was recently shown that IL-3 deprivation induced autophagy in an IL-3-dependent myeloid cell line, but death was not dependent on autophagy, because its inhibition actually enhanced cell death.[14] Thus, IL-3 deprivation is similar to depletion of amino acids, in terms of creating a lack of something necessary for cell survival. Depletion of IL-3 is known to downregulate glucose uptake, eventually leading to an energy crisis[14] that causes the activation of autophagy so that cells can obtain energy. Therefore, the inhibition of autophagy under conditions of IL-3 deprivation or amino-acid depletion enhances cell death due to exacerbation of the energy crisis or shortage of essential components. On the other hand, when Bax/Bak-deficient cells are exposed to etoposide or staurosporine, an

energy crisis may not occur, judging from the observation that inhibition of autophagy improves cell viability. Thus, autophagy is activated in response to etoposide or staurosporine and leads to death by a totally different mechanism to that of autophagy related to cell survival. We think that autophagy may be activated in two different ways, which are (1) by depletion of factors that are necessary for cell survival and (2) by exposure to cytotoxic drugs or other stimuli that do not cause an energy crisis.

To explain this difference in the response, we hypothesize the existence of a specific signaling molecule 'X' that eventually causes the upregulation of Atg5 and Atg6 (Figure 6). 'X' is probably activated by several cytotoxic stimuli, such as etoposide and staurosporine, but not by depletion of amino acids, serum, or lymphokines such as IL-3, although all of these stimuli can trigger apoptosis in normal cells. Detection of 'X' will be crucial for a better understanding of the molecular basis of the signaling pathway involved in autophagic death, as well as for providing a specific marker that allows us to readily distinguish autophagic death from autophagy (survival). Autophagic death is clearly seen after exposure to cytotoxic drugs only when apoptosis is inhibited. However, there might be a signal that specifically activates the autophagic death program *in vivo*, probably through 'X', without having an effect on the apoptotic machinery.

Signaling Pathway: Possible Involvement of Bcl-2 and JNK

Information concerning the molecular basis of autophagic death is extremely limited. All we currently know is that this form of cell death is dependent on autophagy proteins that are involved in the formation of autophagosomes.[8,9] Consistent with the process of autophagy, Atg6 acts upstream of Atg5 during autophagic death.[8]

The following questions remain to be answered: (1) How is the autophagic process activated by cytotoxic stimuli? (2) How are the processes of autophagy (related to cell survival) and autophagic death regulated? (3) Do autophagosomes sequester specific targets that lead to cell death? (4) Why does autophagy sometimes promote cell survival, but causes death under other circumstances?

One intriguing finding is that the autophagic death of Bax/Bak-deficient MEFs requires the antiapoptotic proteins Bcl-x_L or Bcl-2[8] (also refer to Figure 4). Certainly, it is necessary to determine whether this observation also applies to other cells. As Atg6 is required for the autophagic death of Bax/Bak-deficient cells[8] and because Bcl-2/Bcl-x_L (but not Bax/Bak) binds to Atg6 (also called Beclin 1 that was initially identified as a Bcl-2-binding protein),[18] Bcl-2/Bcl-x_L might influence the creation of autophagosomes at least partly via regulation of Atg6. This notion is supported by the finding that an cytotoxic drug-induced increase of Atg5–Atg12 in Bax/Bak-deficient MEFs (which is regulated by Atg6) is markedly reduced by silencing of Bcl-x.[8] This hypothesis needs to be tested by using mutants of Bcl-2/Bcl-x_L and Beclin 1 that cannot interact with each other.

Lenardo's group has shown that autophagic death of L929 cells is dependent on JNK,[9] although they did not determine how JNK and the autophagy machinery were linked. We have confirmed a crucial role of JNK activation in the autophagic death of Bax/Bak-deficient MEFs (our unpublished results), while autophagy that promotes cell survival does not depend on JNK (our unpublished results). Therefore, activation of JNK might be a useful starting point for investigating the signaling pathway of autophagic death.

Role of Autophagic Death in Physiological and Pathological Cell Death

It has been suggested that autophagic death may play a role in both physiological and pathological cell death. This issue has been addressed by some recent reviews.[19,20] However, an important point to remember is that the existing evidence about the role of autophagic death in physiological and pathological cell death processes is rather weak, because it is mainly based on morphologic studies. Therefore, these findings are not discussed here – readers who are interested should refer to the above-mentioned reviews. In this context, discussion of a few points might be useful in terms of setting the direction for future studies on autophagic death.

Autophagic death might be a phylogenetically ancient process, because 'morphologically defined' autophagic death is observed in lower eukaryotes such as nematodes, flies, and slime molds.[21–24] Since genetic manipulation of all these organisms is possible, compelling evidence about the role of autophagic death in PCD should be obtained in near future. 'Morphologically defined' autophagic death has been described in the field of developmental biology. It seems that autophagic death is common during tissue remodeling processes, such as metamorphosis in insects and organ morphogenesis during development.[7] Since autophagy can provide essential nutrients necessary for cells to survive, it is conceivable that autophagy may be used to provide nutrients to other cells in multicellular organisms. This could be an economical way for the remaining cells to reuse the components of organs or tissues that are removed during tissue remodeling and organ morphogenesis. What advantages might autophagic death have over apoptotic death as a form of PCD? It is conceivable that some PCD paradigms may destroy a massive number of cells, so that phagocytes might not be able to remove all of the apoptotic cells and debris. In contrast, the autophagic process could reduce the workload of phagocytes. At any rate, we still need to provide compelling evidence of the direct role of autophagic death in the physiological settings. Since mice are now available with deficiency of autophagy genes, such as Atg6-, Atg5- or Atg7-deficient mice,[25–28] we could utilize such animals to verify that autophagic death plays a role in physiological PCD. Since yeast cells do not possess highly similar apoptotic machinery, but still die in the presence of cytotoxic drugs, it might be of interest to examine whether yeast cells undergo autophagic death.

It is very well established that the inhibition of apoptosis is a critical event in tumorigenesis. Elimination of cancer cells might not only occur via apoptosis but could also be mediated by other forms of cell death such as autophagic death. Some recent observations indicate that a decline of autophagic

activity is related to tumorigenesis.[25,26,29,30] Most importantly, it has been reported that heterozygosity of the *atg6* gene significantly increases tumor development in mice.[25,26] Since it has been shown that autophagy plays a role in antigen presentation, this process might be important for the presentation of tumor antigens[31] and inhibition of autophagy may allow tumor cells to escape immune surveillance. Alternatively, it has been shown that inhibition of autophagy increases the mutation rate in yeasts, although the underlying mechanism is unknown. If this also occurred in mammalian cells, an increase of the mutation rate by inhibition of autophagy in *atg6* heterozygous mice would enhance tumor formation. It has also been suggested that inhibition of autophagy may increase cell proliferation.[26] However, there is also the possibility that autophagic death eliminates tumor cells, so its inhibition would facilitate tumorigenicity as does the inhibition of apoptosis.

To better understand the pathology and the potential therapeutic strategies for human diseases that are characterized by enhanced cell death, it is crucial to elucidate the death mechanism(s) directly involved in each of these diseases. Cell death associated with human diseases could occur via apoptosis, necrosis, autophagic death, or other mechanisms that are currently unknown. Treatment should depend on the actual mechanism of cell death that plays a role in disease onset. There have been some reports suggesting the involvement of autophagic death in various diseases, since cells with autophagic features are found in the lesions of some neurodegenerative diseases, such as Parkinson disease and Alzheimer disease,[7,32,33] as well as in some forms of myopathy.[33,34,35]

Conclusion and Future Prospects

PCD does not seem to be confined to apoptosis. Recent studies,[8,9] although based on nonphysiological models, indicate that cells possess a mechanism of PCD that is associated with the formation of autophagosomes and depends on autophagy proteins – this is the true meaning of autophagic PCD. Although it will not be easy, it is now important to determine whether and to what extent autophagic death is involved when PCD occurs *in vivo*. It is also crucial to determine how apoptosis and autophagic death are regulated. Since there is a distinct possibility that autophagic death is involved in various human diseases, it would be invaluable to understand the molecular basis of autophagic death, so that new therapeutic strategies can be developed. Investigation of autophagic death is still in its infancy, but recently described culture systems,[8,9] that allow autophagic death to be analyzed in detail, should be useful tools for studying this form of cell death under physiological and pathological conditions.

Acknowledgements

The work performed in my laboratory was supported in part by a grant for Center of Excellence Research, a grant for the 21st century COE Program, a grant for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and by a grant for Research on Dementia and Fracture from the Ministry of Health, Labor, and Welfare of Japan. We thank Ms Nao Tsujimoto for editorial assistance.

References

1. Schweichel JU and Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7: 253–266
2. Ohsumi Y (2001) Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell. Biol. 2: 211–216
3. Huang WP and Klionsky DJ (2002) Autophagy in yeast: a review of the molecular machinery. Cell Struct. Funct. 27: 409–420
4. Mizushima N, Ohsumi Y and Yoshimori T (2002) Autophagosome formation in mammalian cells. Cell Struct. Funct. 27: 421–429
5. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI and Deretic V (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 119: 753–766
6. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N and Sasakawa C (2005) Escape of intracellular Shigella from autophagy. Science 307: 727–731
7. Bursch W (2001) The autophagosomal–lysosomal compartment in programmed cell death. Cell Death Differ. 8: 569–581
8. Shimizu S, Kanaseki T, Mizushima N, Mizuta K, Arakawa-Kobayashi S, Thompson CB and Tsujimoto Y (2004) A role of Bcl-2 family of proteins in non-apoptotic programmed cell death dependent on autophagy genes. Nat. Cell Biol. 6: 1221–1228
9. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH and Lenardo MJ (2004) Regulation of an ATG7-Beclin 1 program of autophagic cell death by caspase-8. Science 304: 1500–1502
10. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR and Thompson CB (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell 6: 1389–1399
11. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292: 727–730
12. Yue Z, Horton A, Bravin M, DeJager PL, Selimi F and Heintz N (2002) A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. Neuron 35: 921–933
13. Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, Cho DH, Choi B, Lee H, Kim JH, Mizushima N, Oshumi Y and Jung YK (2005) Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. J. Biol. Chem. 280: 20722–20729
14. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T and Thompson CB (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell 120: 237–248
15. Lee CY and Baehrecke EH (2001) Steroid regulation of autophagic programmed cell death during development. Development 128: 1443–1455
16. Martin DN and Baehrecke EH (2004) Caspases function in autophagic programmed cell death in *Drosophila*. Development 131: 275–284
17. Lee CY, Cooksey BA and Baehrecke EH (2002) Steroid regulation of midgut cell death during *Drosophila* development. Dev. Biol. 250: 101–111
18. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B and Levine B (1998) Protection against fatal Sindbis virus encephalitis by Beclin, a novel Bcl-2-interacting protein. J. Virol. 72: 8586–8596
19. Gozuacik D and Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23: 2891–2906
20. Levine B and Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell 6: 463–477
21. Cornillon S, Foa C, Davoust J, Buonavista N, Gross JD and Golstein P (1994) Programmed cell death in *Dictyostelium*. J. Cell Sci. 107 (Part 10): 2691–2704
22. Olie RA, Durrieu F, Cornillon S, Loughran G, Gross J, Earnshaw WC and Golstein P (1998) Apparent caspase independence of programmed cell death in *Dictyostelium*. Curr. Biol. 8: 955–958
23. Hall DH, Gu G, Garcia-Anoveros J, Gong L, Chalfie M and Driscoll M (1997) Neuropathology of degenerative cell death in *Caenorhabditis elegans*. J. Neurosci. 17: 1033–1045

24. Baehrecke EH (2003) Autophagic programmed cell death in *Drosophila*. Cell Death Differ. 10: 940–945
25. Yue Z, Jin S, Yang C, Levine AJ and Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. USA 100: 15077–15082
26. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G and Levine B (2003) Promotion of tumorigenesis by heterozygous disruption of the Beclin 1 autophagy gene. J. Clin. Invest. 112: 1809–18020
27. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T and Mizushima N (2004) The role of autophagy during the early neonatal starvation period. Nature 432: 1032–1036
28. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K and Chiba T (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. 169: 425–434
29. Kisen GO, Tessitore L, Costelli P, Gordon PB, Schwarze PE, Baccino FM and Seglen PO (1993) Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. Carcinogenesis 14: 2501–2505
30. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H and Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 402: 672–676
31. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T and Munz C (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307: 593–596
32. Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, Mouatt-Prigent A, Ruberg M, Hirsch EC and Agid Y (1997) Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol. Histopathol. 12: 25–31
33. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. J. Neurochem. 83: 481–489
34. Kaneda D, Sugie K, Yamamoto A, Matsumoto H, Kato T, Nonaka I and Nishino I (2003) A novel form of autophagic vacuolar myopathy with late-onset and multiorgan involvement. Neurology 61: 128–131
35. Kalimo H, Savontaus ML, Lang H, Paljarvi L, Sonninen V, Dean PB, Katevuo K and Salminen A (1988) X-linked myopathy with excessive autophagy: a new hereditary muscle disease. Ann. Neurol. 23: 258–265

International weekly journal of science

nature

Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death

Takashi Nakagawa[*1,2,4], Shigeomi Shimizu[*1,4], Tetsuya Watanabe[*3], Osamu Yamaguchi[*3], Kinya Otsu[*3], Hirotaka Yamagata[*1], Hidenori Inohara[*2], Takeshi Kubo[*2] & Yoshihide Tsujimoto[*1,4]

[*1]*Laboratory of Molecular Genetics, Department of Post-Genomics and Diseases,*
[*2]*Department of Otolaryngology and Sensory Organ Surgery and*
[*3]*Department of Internal Medicine and Therapeutics, Osaka University Medical school, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[*4]*Solution-Oriented Research for Science and Technology (SORST), Japan Science and Technology Corporation, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

Reprinted from Nature, Vol. 434, No. 7033, pp. 652–658, 31 March 2005

Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death

Takashi Nakagawa[1,2,4], Shigeomi Shimizu[1,4], Tetsuya Watanabe[3], Osamu Yamaguchi[3], Kinya Otsu[3], Hirotaka Yamagata[1], Hidenori Inohara[2], Takeshi Kubo[2] & Yoshihide Tsujimoto[1,4]

[1]*Laboratory of Molecular Genetics, Department of Post-Genomics and Diseases,* [2]*Department of Otolaryngology and Sensory Organ Surgery and* [3]*Department of Internal Medicine and Therapeutics, Osaka University Medical school, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

[4]*Solution-Oriented Research for Science and Technology (SORST), Japan Science and Technology Corporation, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

Mitochondria play an important role in energy production, Ca^{2+} homeostasis and cell death. In recent years, the role of the mitochondria in apoptotic and necrotic cell death has attracted much attention[1,2]. In apoptosis and necrosis, the mitochondrial permeability transition (mPT), which leads to disruption of the mitochondrial membranes and mitochondrial dysfunction, is considered to be one of the key events, although its exact role in cell death remains elusive. We therefore created mice lacking cyclophilin D (CypD), a protein considered to be involved in the mPT, to analyse its role in cell death. CypD-deficient mice were developmentally normal and showed no apparent anomalies, but CypD-deficient mitochondria did not undergo the cyclosporin A-sensitive mPT. CypD-deficient cells died normally in response to various apoptotic stimuli, but showed resistance to necrotic cell death induced by reactive oxygen species and Ca^{2+} overload. In addition, CypD-deficient mice showed a high level of resistance to ischaemia/reperfusion-induced cardiac injury. Our results indicate that the CypD-dependent mPT regulates some forms of necrotic death, but not apoptotic death.

The mitochondrial permeability transition (mPT) is a regulated Ca^{2+}-dependent increase in the permeability of the mitochondrial membrane, which results in a loss in mitochondrial membrane potential ($\Delta\Psi$), mitochondrial swelling and rupture of the outer membrane[3]. The mPT is thought to occur after the opening of a channel, termed the permeability transition pore, which putatively consists of a voltage-dependent anion channel, an adenine nucleotide translocator, CypD, and some other molecule(s)[4]; however, an essential role for the adenine nucleotide translocator in the mPT is a matter of recent controversy[5,6].

CypD is a mitochondrial member of the cyclophilin family of peptidyl prolyl-*cis*, *trans*-isomerases (PPIases) and has a crucial role in protein folding[7]. It has been suggested that CypD is involved in regulating the mPT, on the basis of the observation that cyclosporin A (CsA), a specific inhibitor of cyclophilin family activity, blocks the mPT[8]. A CsA-insensitive mPT has also been suggested, although the molecular mechanism is completely unknown[9]. It has been shown that some forms of apoptosis are significantly inhibited by CsA, suggesting a role for CsA-sensitive mPT in apoptosis[2,4]. The mPT is also implicated in the remodelling of mitochondrial structure with mobilization of cytochrome *c* stores in cristae during apoptosis[10]. To determine whether CypD has a crucial role in the CsA-sensitive mPT, and to investigate whether the mPT is a key regulator of cell death, we created CypD-deficient mice by gene targeting (see Supplementary Fig. 1a–c). The absence of cyclophilin D protein in CypD-deficient mice was verified by western blotting (see Fig. 1a and Supplementary Fig. 1d). CypD-deficient mice were born at the expected mendelian frequency, developed normally, and did not have any detectable phenotypic anomalies.

To examine the role of CypD in the mPT, mitochondria were isolated from the livers of CypD-deficient mice and control littermates. The mitochondria showed no significant change in respiration rate in the absence of CypD (see Supplementary Fig. 2). As shown in Fig. 1a, PPIase activity was absent in CypD-deficient mitochondria, but not in control mitochondria, indicating that CypD is the major PPIase in the mitochondria. When control mitochondria were treated with 50 μM Ca^{2+}, the mPT occurred, as shown by mitochondrial swelling (Fig. 1b) and loss of $\Delta\Psi$ (Fig. 1c). These phenomena were not observed in CypD-deficient mitochondria (Fig. 1b, c). To examine the extent of mPT inhibition as a consequence of CypD-deficiency, successive doses of Ca^{2+} were added to the mitochondria. $\Delta\Psi$ was lost after two additions of 50 μM Ca^{2+} to control mitochondria, but it was still maintained after seven additions of Ca^{2+} to CypD-deficient mitochondria (Fig. 1d). We investigated whether the absence of Ca^{2+}-induced mPT in CypD-deficient mitochondria was due to disturbance of Ca^{2+} uptake. As shown in Fig. 1e, the extra-mitochondrial Ca^{2+} concentration increased transiently, and rapidly returned to basal levels after each successive addition of Ca^{2+} to CypD-deficient mitochondria, indicating normal Ca^{2+} uptake by the mitochondria. Up to concentrations of 500 μM Ca^{2+}, most of the Ca^{2+} was taken up by CypD-deficient mitochondria (Fig. 1e, f). Next, we analysed CypD-deficient mitochondria in the presence of much higher concentrations of Ca^{2+}, which can induce CsA-insensitive mPT[9]. Addition of more than 1 mM Ca^{2+} induced swelling, collapse of $\Delta\Psi$, and impaired Ca^{2+} uptake even in CypD-deficient mitochondria (Fig. 1f and Supplementary Fig. 3), and all of these events were insensitive to CsA, even when it was added to CypD-deficient mitochondria (see Supplementary Fig. 3). Taken together, these results suggest that the mPT induced by low doses of Ca^{2+} is completely inhibited in CypD-deficient mitochondria, and that CypD is not involved in the CsA-insensitive increase in membrane permeability induced by high doses of Ca^{2+}. Furthermore, the addition of other mPT inducers, such as H_2O_2 and atractyloside, did not trigger the mPT in CypD-deficient mitochondria (see Supplementary Fig. 4).

In many forms of apoptosis, BH3-domain-containing proteins of the Bcl-2 family ('BH3-only' proteins) transduce apoptotic signals to the mitochondria, and induce cytochrome *c* release in a Bax/Bak-dependent manner[11]. Whether the CsA-sensitive mPT is involved in apoptotic cytochrome *c* release is controversial[12–15]. To address this question, we added recombinant Bid (rBid), one of the BH3-only proteins, to CypD-deficient and control mitochondria. As shown in Fig. 1g (top panel), CypD deficiency had no effect on rBid-induced cytochrome *c* release, which was different from the result in Bak-deficient mitochondria (Fig. 1g, bottom panel). Bad (another BH3-only protein, data not shown) and rBax (Fig. 1g; second panel), were also found to induce cytochrome *c* release equally in both CypD-deficient and control mitochondria. In contrast, Ca^{2+}-induced cytochrome *c* release was markedly reduced in CypD-deficient mitochondria (Fig. 1g; third panel). Interestingly, Bak deficiency did not have any effect on Ca^{2+}-induced cytochrome *c* release (Fig. 1g, bottom panel). Together, these data indicate that the mPT is involved in cytochrome *c* release induced by Ca^{2+}, but not by pro-apoptotic BH3-only proteins or Bax.

The results described above raise the possibility that the mPT might be involved in cell death due to mPT inducers (including Ca^{2+} overload and reactive oxygen species), but is not involved in the common apoptotic pathway to which BH3-only proteins and Bax/Bak are committed. We first examined the responses of CypD-deficient cells to these death stimuli. As shown in Fig. 2a, control and CypD-deficient thymocytes underwent comparable

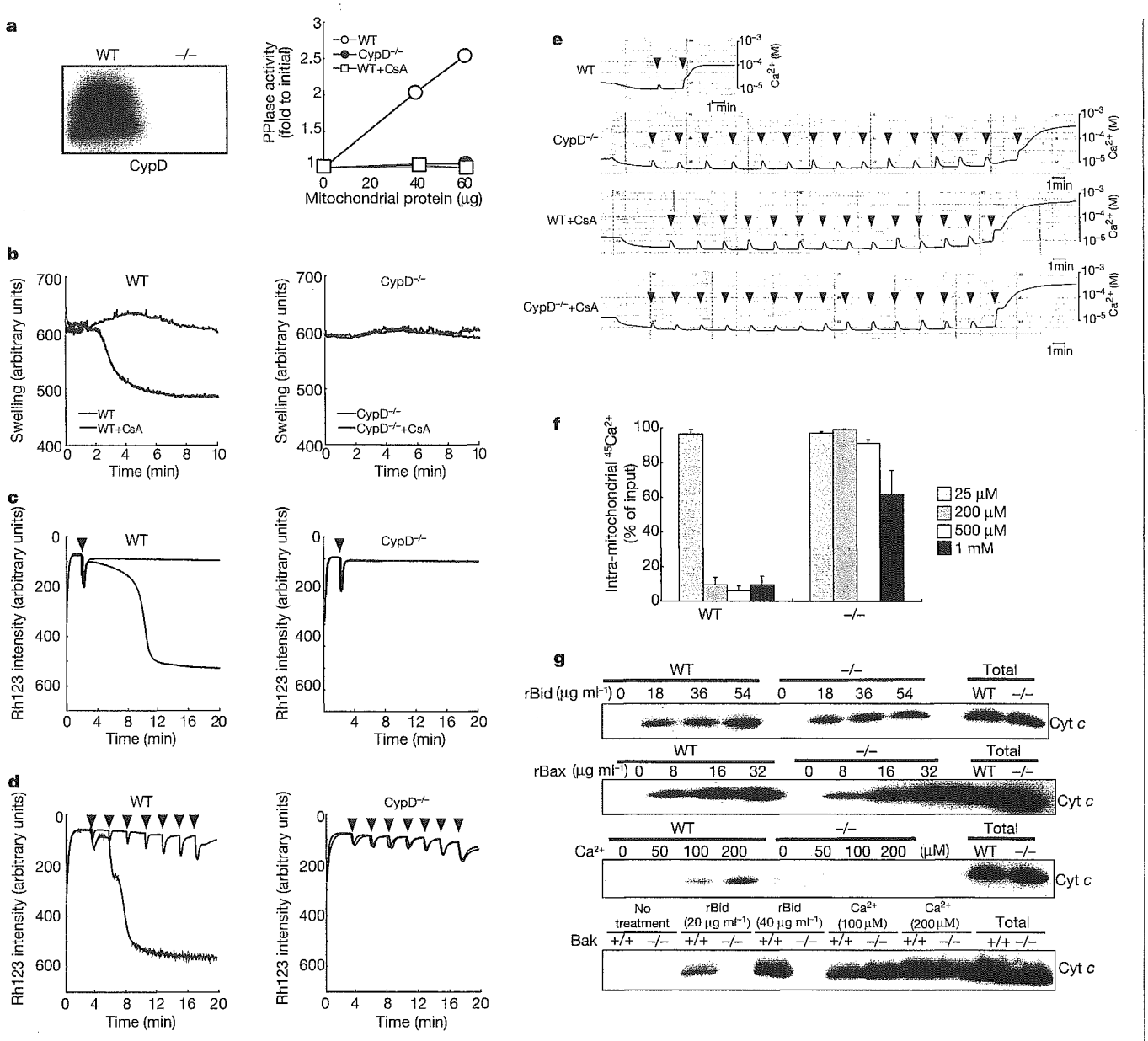

Figure 1 Absence of mPT in CypD-deficient ($CypD^{-/-}$) mitochondria. **a**, Absence of CypD protein and PPIase activity in $CypD^{-/-}$ mitochondria. WT, wild type. **b**, **c**, Absence of mPT in $CypD^{-/-}$ mitochondria. Isolated wild-type (WT, left column) or $CypD^{-/-}$ (right column) mitochondria were incubated with 50 μM Ca^{2+} in the presence (pink) or absence (blue) of 1 μM CsA, and monitored for (**b**) swelling (by light scatter) or (**c**) $\Delta\Psi$ (by Rh123 intensity), see Methods. Loss of $\Delta\Psi$ causes release of Rh123 from the mitochondria, resulting in increased Rh123 intensity. **d**, **e**, CypD deficiency prevents Ca^{2+}-induced $\Delta\Psi$ loss without altering Ca^{2+} uptake. Isolated mitochondria were successively treated with 50 μM Ca^{2+} (indicated by arrowheads) in the presence (pink) or absence (blue) of 1 μM CsA, and $\Delta\Psi$ (**d**) and extra-mitochondrial Ca^{2+} (**e**) were monitored. **f**, Accumulation of Ca^{2+} in the mitochondria as a result of CypD deficiency. Mitochondria were incubated with the indicated concentrations of $^{45}Ca^{2+}$ for 25 min and intra-mitochondrial Ca^{2+} was measured. Data shown as mean ± s.e.m. **g**, Lack of Ca^{2+}-induced, but not rBid- and rBax-induced, cytochrome *c* release in $CypD^{-/-}$ mitochondria. WT, $Bak^{-/-}$ and $CypD^{-/-}$ mitochondria were incubated with Ca^{2+}, rBid, and rBax at the indicated concentrations. After 30 min, samples were centrifuged and aliquots of supernatants were subjected to western blot analysis for cytochrome *c*. 'Total' represents the total amount of cytochrome *c* found in an equivalent aliquot of the mitochondria.

levels of apoptosis when exposed to various apoptotic stimuli. Similar findings were also obtained when murine embryonic fibroblast cells (MEFs) and hepatocytes from CypD-deficient mice were treated with various apoptotic reagents (Fig. 2b, c), and when these cells were transfected with DNA encoding Bax or a truncated form of Bid (tBid; Fig. 2d, e). Moreover, apoptotic death of intestinal epithelial cells also occurred equally in control and CypD-deficient mice subjected to X-ray irradiation (Fig. 2f). These results indicate that CypD-dependent (CsA-sensitive) mPT is not involved in the common apoptotic pathway.

Next, we tested control and CypD-deficient MEFs for cell death after exposure to H_2O_2. CypD-deficient MEFs were more resistant to H_2O_2-induced cell death than control cells as assessed by a CTB (cell titre blue) assay, which measures the metabolic activity of viable cells (Fig. 3a), and by Annexin-V staining (data not shown). H_2O_2-induced cell death is predominantly due to necrosis, based on the following observations: a lack of caspase activation (Fig. 3b), no effect of a caspase inhibitor (Fig. 3a), early disruption of the plasma

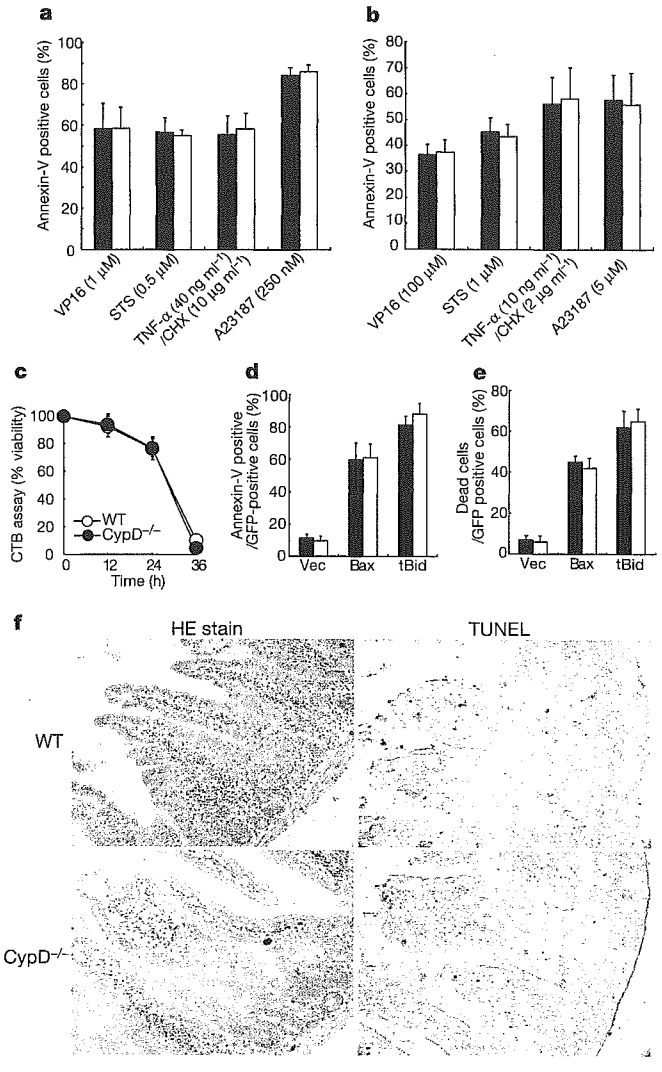

Figure 2 No resistance of Cyp D$^{-/-}$ cells to multiple apoptotic stimuli **a**, **b**, Susceptibility of primary thymocytes and MEFs to various apoptotic stimuli. Wild-type (black) and Cyp D$^{-/-}$ (grey) thymocytes (**a**) and MEFs (**b**) were exposed to apoptosis-inducing reagents for 24 h, and apoptotic cells were assessed by Annexin-V staining. Reagents: etoposide (VP16), staurosporin (STS), tumour-necrosis factor-α (TNF-α) + cycloheximide (CHX), A23187. **c**, Susceptibility of primary hepatocytes to 10 μM STS. Wild-type and CypD$^{-/-}$ hepatocytes were treated with STS for 36 h and cell death was assessed using the CTB assay. Data shown as mean ± s.e.m. **d**, **e**, No effect of CypD deficiency on Bax- or tBid-induced death of MEFs and hepatocytes. Immortalized MEFs (**d**) and primary cultured hepatocytes (**e**) were transiently transfected with DNA for Bax (1 μg) or tBid (1 μg) plus enhanced green fluorescent protein (EGFP, 0.5 μg) for 24 h, and cells were stained with Cy3-conjugated Annexin-V. The percentage of Annexin-V positive cells was calculated relative to all GFP-positive cells. Wild type, black; CypD$^{-/-}$, grey. Data shown as mean ± s.e.m. **f**, No effect of CypD deficiency on X-ray-induced apoptosis in the small intestine. Wild-type and CypD$^{-/-}$ mice were exposed to 10 Gy irradiation. After 72 h, a segment of the small intestine was excised and subjected to haematoxylin-eosin (HE) and TUNEL staining.

membrane (Fig. 3c), and finally, no nuclear or oligonucleosomal DNA fragmentation (Fig. 3c and data not shown). Similar results for H_2O_2-induced cell death were also obtained using CypD-deficient hepatocytes (Fig. 3d and data not shown).

We also examined the effect of CypD on cell death induced by A23187, a Ca^{2+} ionophore. CypD-deficient hepatocytes showed significant resistance to A23187-induced cell death (Fig. 3e). Like H_2O_2-induced cell death, A23187-induced cell death was not accompanied by caspase activation (Fig. 3f), so this type of death was also considered to represent necrotic cell death. These results indicate that CypD (and the CsA-sensitive mPT) is involved in necrotic cell death induced by reactive oxygen species or Ca^{2+} overload.

Suppression of A23187-induced cell death by CypD deficiency seemed likely to be due to inhibition of the mPT. To determine whether A23187-induced mPT is suppressed by CypD deficiency in cells, we monitored mitochondrial $\Delta\Psi$ and Ca^{2+} accumulation in the presence of A23187. Hepatocytes were loaded with the $\Delta\Psi$ markers tetramethylrhodamine methylester (TMRM) or Mito Tracker Orange CMTM Ros, and treated with A23187, after which the fluorescence intensity was monitored by real-time imaging. Addition of A23187 caused rapid loss of $\Delta\Psi$ in control (wild-type) hepatocytes, whereas CypD-deficient hepatocytes maintained $\Delta\Psi$ for much longer periods of time (Fig. 3g–i and Supplementary Fig. 5). The addition of CCCP (carbonyl cyanide m-chlorophenyl-hydrazone), a protonophore, completely dissipated $\Delta\Psi$. Mitochondrial accumulation of Ca^{2+} was investigated using Rhod2-AM[16]. After A23187 treatment, CypD-deficient hepatocytes showed a rapid increase in Rhod2 fluorescence intensity, but wild-type hepatocytes showed only a marginal increase (Fig. 3j–l). The validity of using Rhod2 as an indicator of mitochondrial Ca^{2+} under these conditions was confirmed using Ru360, an inhibitor of the mitochondrial calcium uniporter (Fig. 3l). These results indicate that compared to control hepatocytes, CypD-deficient hepatocytes absorb a larger amount of cytosolic Ca^{2+} into their mitochondria without loss of mitochondrial $\Delta\Psi$; this is consistent with the results obtained using isolated mitochondria, and suggests that A23187 induces CypD-dependent mPT in cells.

Finally, we investigated the role of CypD in ischaemia/reperfusion (I/R) injury, in which disturbance of Ca^{2+} homeostasis and generation of reactive oxygen species have been implicated[17]. Many reports have described a protective effect of CsA against I/R injury[18–21]. First, we investigated whether CypD-deficient mitochondria showed resistance to anoxia/reoxygenation-induced injury, which simulates I/R-induced injury *in vivo*. Isolated mitochondria from control and CypD-deficient mice were subjected to anoxia for 30 min, followed by reoxygenation. Control mitochondria, but not CypD-deficient mitochondria, showed loss of $\Delta\Psi$ (Fig. 4a), swelling (Fig. 4b), leakage of mitochondrial asparatate aminotransferase (mAST) (Fig. 4c), and a severe decrease in respiratory control rate (Fig. 4d), indicating that CypD-deficient mitochondria are more resistant to anoxia/reoxygenation injury than control mitochondria.

We next examined the effect of CypD on cardiac I/R injury, because the heart has high levels of CypD (see Supplementary Fig. 1d). Several functional parameters assessed by echocardiography showed no differences between the resting hearts of control and CypD-deficient mice (see Supplementary Table). Mice were subjected to 30 min of left coronary artery occlusion followed by 2 h of reperfusion. The size of the area at risk, identified by the absence of Evans blue staining, was not significantly different between control and CypD-deficient hearts (Fig. 4f). In control hearts, I/R injury caused significant necrotic damage, as evidenced by a large area of myocardium that was negative for triphenyltetrazolium chloride (TTC) staining (Fig. 4e, g). In CypD-deficient hearts, however, the infarct area was dramatically reduced (Fig. 4e, g). Consistently, lactate dehydrogenase (LDH) release due to disruption of the plasma membrane was almost completely inhibited in CypD-deficient hearts (Fig. 4h). These results indicate that lack of CypD can markedly reduce cardiac I/R injury.

An increase in the permeability of the outer mitochondrial membrane is central to apoptotic signalling, and is directly regu-

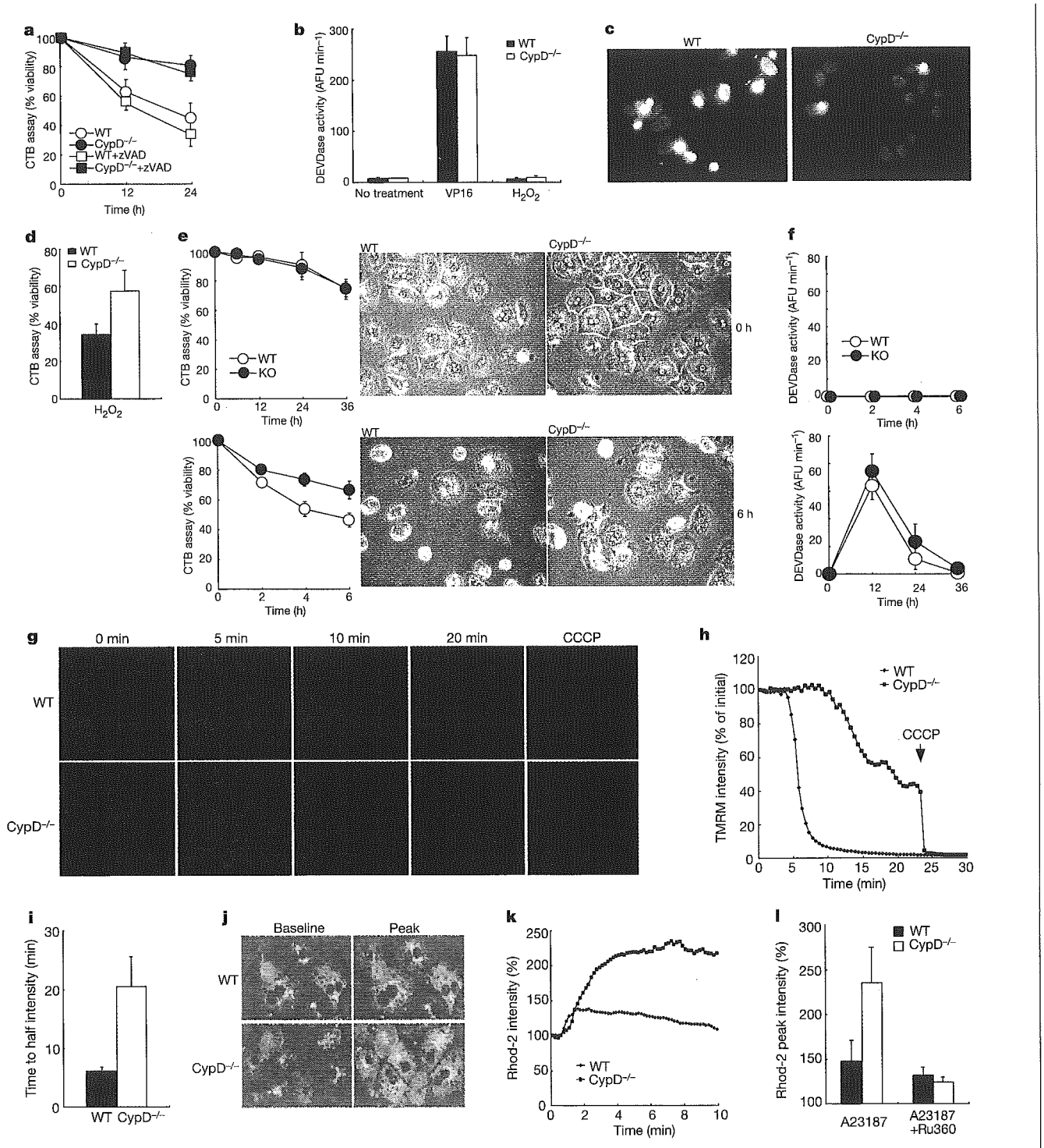

Figure 3 Resistance of CypD$^{-/-}$ cells to necrosis induced by reactive oxygen species and Ca^{2+} overload. **a–d**, Reduction of H_2O_2-induced necrotic cell death by CypD deficiency. Wild-type (WT) and CypD$^{-/-}$ MEFs (**a–c**) and hepatocytes (**d**) were exposed to H_2O_2 (**a–c**, 0.75 mM for 24 h; **d**, 0.5 mM for 4 h), and the extent of cell death was assessed by CTB assay (**a**, **d**). zVAD is a pan-caspase inhibitor. **b**, Caspase activation in wild-type (black) and CypD$^{-/-}$ (grey) hepatocytes after treatment with 0.75 mM H_2O_2 was assessed at 16 h. Treatment with VP16 (100 μM) for 16 h was used as a positive control for caspase activation. Data shown as mean ± s.e.m. **c**, Representative nuclear changes visualized by staining with Hoechst 33342 (blue) and PI (red) at 12 h. **e**, WT (open circles) and CypD$^{-/-}$ (filled circles) hepatocytes left untreated (top panel) or treated with 2 μM A23187 (bottom panel), measured using the CTB assay. Data shown as mean ± s.e.m. Phase contrast microscopy images at 0 h and 6 h are shown. **f**, Assessment of caspase activation in response to 2 μM A23187 (upper panel); treatment with 40 ng ml^{-1} TNF-α + 10 μg ml^{-1} CHX was used as a positive control for caspase activation (lower panel). Data shown as mean ± s.e.m. **g–i**, Reduced $\Delta\Psi$ loss in CypD$^{-/-}$ hepatocytes (preloaded with TMRM) treated with 10 μM A23187. TMRM fluorescence intensity was monitored by laser scanning confocal microscopy. Representative real-time images (**g**), average TMRM intensity of individual WT (blue) and CypD$^{-/-}$ (pink) cells (**h**), and the half-life time of the fluorescence intensity of individual cells (**i**) are shown. Data shown as mean ± s.d. The arrow in (**h**) indicates the addition of 5 μM CCCP, which completely dissipated $\Delta\Psi$. **j–l**, Increased A23187-induced mitochondrial Ca^{2+} uptake by CypD$^{-/-}$ hepatocytes. Cells were loaded with Rhod2-AM and treated with 10 μM A23187. Rhod2 fluorescence intensity was monitored by laser scanning confocal microscopy. Representative images of baseline and peak fluorescence are shown (**j**). Average (**k**) and peak (**l**) fluorescence intensities of cells are shown (mean ± s.d.). In (**l**), hepatocytes were also loaded with Rhod2-AM in the presence of 1 μM Ru360.

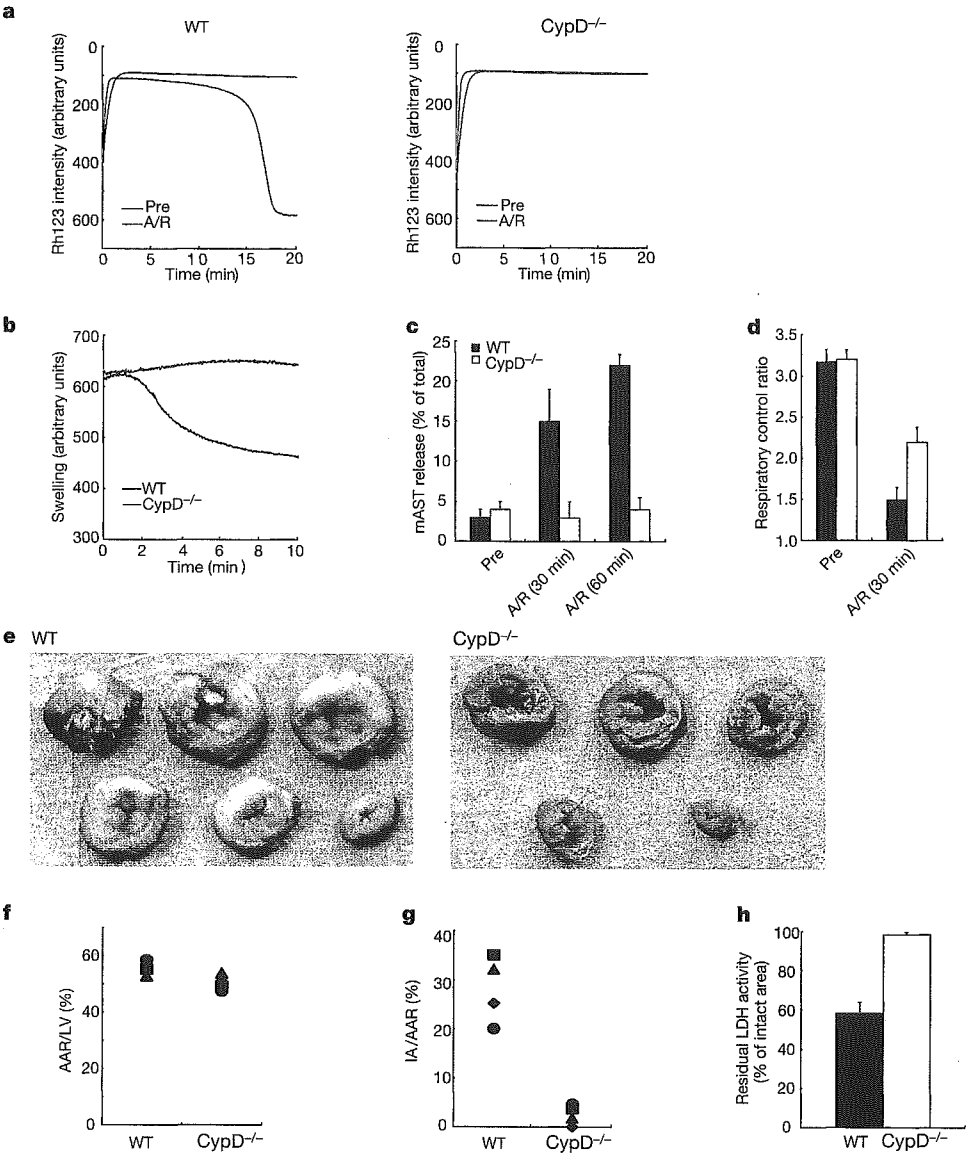

Figure 4 Prevention of cardiac ischaemia/reperfusion injury in CypD$^{-/-}$ mice. **a–d**, Absence of mitochondrial damage induced by anoxia/reoxygenation (A/R) in CypD$^{-/-}$ mitochondria. Isolated mitochondria were treated without (Pre) or with anoxia for 30 min followed by reoxygenation for the indicated times. The $\Delta\Psi$ (**a**), swelling (**b**), mAST release (**c**), and respiratory control ratio (state 3/state 4) (**d**) were measured. Data shown as mean ± s.e.m. **e–h**, Reduction of cardiac I/R injury in CypD$^{-/-}$ mice. **e**, Representative slices of a heart subjected to I/R. The slices were double-stained with Evans blue (blue) and TTC (red). The infarct region was not stained (white). **f**, The area at risk/left ventricle (AAR/LV) and (**g**) the infarct area/area at risk (IA/AAR). Data are shown for four independent experiments. **h**, The ratio of residual LDH activity in the IA/non-ischaemic area. Data shown as mean ± s.d.

lated by the Bcl-2 family of proteins[1]. It has been suggested that the mPT plays a role in apoptotic mitochondrial membrane permeabilization[22]. However, we show here that cytochrome *c* release induced by Bid and Bax is not blocked by CypD deficiency and that CypD deficiency does not affect many forms of apoptotic cell death, indicating that the CypD-dependent mPT does not play a significant role in apoptosis in general; however, this does not exclude the possibility that certain forms of apoptosis are mediated by the mPT, and thereby inhibited by CsA. On the other hand, CypD deficiency blocks Ca^{2+}-induced and oxidative stress-induced cytochrome *c* release from isolated mitochondria and also prevents necrotic cell death induced by these stimuli; this indicates that the CypD-dependent mPT is a critical event in some forms of cellular necrosis. Notably, overexpressed CypD can induce necrosis[23].

We showed that lack of CypD markedly suppresses cardiac I/R injury (which mimics cardiac infarction). In I/R injury, production of reactive oxygen species and Ca^{2+} overload are known to be key events[17]. Our results indicate that CypD-deficient mitochondria can accumulate excess Ca^{2+} without loss of $\Delta\Psi$ and that they also tolerate reactive oxygen species-induced damage. Consistently, CypD-deficient hepatocytes accumulated more Ca^{2+} than wild-type hepatocytes, and were significantly more resistant to A23187- and H_2O_2-induced death. Thus, the CypD-dependent mPT is a critical event in I/R injury, suggesting that CypD and the mPT may be important therapeutic targets for preventing myocardial infarction. □