Apoptosis is characterized by a series of distinct morphological and biochemical changes which seem to be well conserved through evolution. One of the best described proapoptotic genes, CED-3, encodes a Caenorhabditis elegans protein that is highly homologous to mammalian interleukin-1β converting enzyme (ICE), the first identified member of a new class of cysteine proteases with a near absolute specificity for aspartic acid in the S1 subsite (5–8).

Recent work has indicated the involvement of a protease belonging to the ICE/CED-3 family in Fas-mediated apoptosis (9–11). We have previously used a reconstituted vitro system (12, 13) containing cytosolic extracts from Jurkat T cells undergoing Fas-induced apoptosis to partially purify a protease that promotes apoptotic changes in isolated nuclei (14). In the absence of 1,4-dithiothreitol (DTT), this proteolytic activity was inhibited by several serine protease inhibitors, iodoacetamide and VAD-FMK, while E64 was not inhibitory (14). This inhibitor profile is consistent with the protease being a member of the ICE/CED-3 family (6, 15). In the present work we demonstrate that this protease, which appears to play a crucial role in Fas-induced apoptosis, is not ICE itself but another member of the ICE/CED-3 family, namely CPP32/apopain.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The human leukemic T cell line, J urkat, was obtained from the ATCC (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin in an atmosphere of 5% CO2 in air at 37°C. Anti-human Fas antibody (IgM, clone CH-11) was purchased from AMS Biotechnology, Sweden. RPMI 1640 medium, fetal calf serum, glutamine, penicillin, streptomycin, and low-melting-point agarose were obtained from Life Technologies, Inc. (Paisley, Scotland, UK). All other chemicals were from local suppliers and of the highest available grade of purity.

Extraction of J urkat T Cells for the Measurement of PARP- and Pro-IL-1β Cleavage Activity, DNA Fragmentation, and Immunoblot Analysis—Jurkat T cells (2 × 107 cells/ml) were incubated at 37°C with the isolated protease from Jurkat cells undergoing Fas-induced apoptosis in the presence or absence of inhibitors. After 70 min, the reaction was stopped and the samples were divided for analysis by field inversion gel electrophoresis and conventional agarose electrophoresis as described (14, 16). The rest of the cells was lysed with four cycles of freezing and thawing. The cell lysates were centrifuged for 15 min at 20,000 × g to pellet the membranous fraction. A further centrifugation step for 30 min at 120,000 × g yielded a supernatant fraction consisting mainly of cytosolic proteins. The [35S]PARP and [35S]pro-IL-1β cleavage activity of the extracts obtained was measured as described elsewhere (6, 17). Immunoblot analysis of the extracts after SDS-gel electrophoresis was done using rabbit polyclonal antibodies specific for the p12 and p17 subunits of CPP32/apopain.

Isolation of Nuclei and Incubation Conditions—Rat thymocyte nuclei were prepared as described (14). Using a recently published in vitro system (13, 14, 18), thymocyte nuclei were incubated in S-buffer (see above) plus 2 mM DTT at 37°C with the isolated protease from Jurkat cells undergoing Fas-induced apoptosis in the presence or absence of inhibitors. After 70 min, the reaction was stopped and the samples were divided for analysis by field inversion gel electrophoresis and conventional agarase electrophoresis as described in Ref. 14.

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**CPP32/ Apopain in Fas-mediated Apoptosis**

Measurement of the Inhibition by Specific Peptide Aldehydes of DNA Fragmentation and Cell Death—Jurkat T cells (10 × 10^6/ml) were preincubated at 37°C for 1 h with 120 μM Ac-YVAD-CHO, 120 μM Ac-DEVD-CHO, or 120 μM Ac-DEVD-COOH before incubation with anti-Fas antibody (0.75 μg/ml) for an additional 2 h. Aliquots of the incubated cells were used to monitor membrane integrity by trypan blue exclusion. After 2 h of incubation with anti-Fas antibody, the cells were isolated by centrifugation, washed, and analyzed for DNA fragmentation as described (16).

**RESULTS AND DISCUSSION**

In cytosolic extracts prepared from anti-Fas-treated Jurkat T cells, elevation of a PARP cleavage activity is an early event first detectable 15 min after adding an anti-Fas antibody to the cells (Fig. 1a). By comparison, fragmentation of nuclear DNA into high molecular weight fragments starts at about 30 min (Fig. 1d), while the formation of internucleosomal DNA fragments cannot be detected earlier than 45 min after antibody addition (not shown). We could not detect any proteolytic activity capable of cleaving pro-IL-1β in extracts taken at different time points during incubation with anti-Fas antibody (Fig. 1b), even at high concentrations of the extracts (not shown).

Furthermore, these cells contained no ICE protein that could be recognized by immunoblotting or immunoprecipitation (not shown). This clearly argues against the involvement of ICE itself in Fas-mediated apoptosis in Jurkat T cells. However, the early elevation of a proteolytic activity that cleaves PARP (Fig. 1a) implies the involvement of another enzyme of the ICE family, apopain (17) (prICE/CPP32 (18, 19)), an ICE-like protease reported to cleave PARP (17, 18). This protease is synthesized as a 32-kDa precursor (p32) that is proteolytically activated to form a mature enzyme composed of 17-kDa (p17) and 12-kDa (p12) subunits (17). ImmunobLOTS using antibodies specific for the p17 and p12 subunits of apopain show the presence of the dormant 32-kDa precursor in untreated Jurkat T cells (Fig. 1c). Following anti-Fas activation, this precursor was rapidly broken down into active apopain (Fig. 1c) coincident with the onset of the PARP cleavage activity. The precursor was initially processed to p12 and a 20-kDa intermediate (p20) (detectable by 30 min), followed by slower conversion of p20 to p17. Both p20 and p17 have catalytic activity as shown by their ability to be labeled with a biotinylated irreversible DEVD-acetylamidomethyl ketone active site probe of apopain with biotinylation found as early as the 15-min time point (not shown). The proapoptotic protease isolated from anti-Fas-treated Jurkat T cells was found to contain primarily p12 and p17 (Fig. 1c), indicating that apopain was the protease that had been purified. The fact that the 32-kDa CPP32-precursor of apopain is converted so quickly to the subunits that comprise active apopain, with maximal activation of apopain occurring within 60 min after anti-Fas treatment of the cells (Fig. 1a and Table I), might explain why Fas-mediated apoptosis in Jurkat T cells is that much faster than other model systems of apoptosis.

When the partially purified protease (which was unable to cleave pro-IL-1β) was tested for its ability to cleave PARP in the presence of selective inhibitors, the apopain inhibitor Ac-DEVD-CHO (4) (K_i,CPP32 < 1 nM) gave complete inhibition at 5 μM whereas the ICE inhibitor Ac-YVAD-CHO (6) (K_i,ICE = 0.76 nM) was inactive at 5 μM and only partially effective at 50 μM (Fig. 2a). Measurement of the ability of the isolated protease to induce apoptotic changes in thymocyte nuclei using a reconstituted in vitro system revealed similar results. The ICE inhib-

![Fig. 1. Time course of events in anti-Fas-treated Jurkat T cells.](image)

**Table I**

| Enzyme source | K_m (μM) | K_i (nM) |
|---------------|---------|---------|
| Apopain/CPP32 purified from THP.1 cells | 13.9 ± 0.5 | 1.1 ± 0.1 x 10^6 |
| Extracts from anti-Fas-treated Jurkat T cells | 15.0 ± 1.0 | 0.9 ± 0.1 x 10^6 |
| Partially purified protease from Jurkat T cells | 14.4 ± 0.8 | 1.0 ± 0.1 x 10^6 |

*1 unit is defined as the amount of enzyme required to liberate 1 pmol of AMC in 1 min at 25°C using 100 μM substrate.*
showed a viability of DEVD-COOH were without effect (Fig. 3). Since the cells optosis in Jurkat T cells, whereas Ac-YVAD-CHO and Ac-DEVD-CHO at concentrations as high as 50 μM were found to inhibit Fas-induced apoptosis in whole cells, we preincubated Jurkat T cells by the specific tetrapeptide aldehyde inhibitor for apopain/CPP32 as described (17). In other cell types (9, 10), Ac-DEVD-CHO inhibits apoptotic changes in vitro at nanomolar concentrations. Thymocyte nuclei were incubated with the partially purified protease from Jurkat T cells undergoing Fas-induced apoptosis in the absence or presence of AcYVAD-CHO, a specific inhibitor for ICE, or Ac-DEVD-CHO, a specific inhibitor for apopain/CPP32 as described (17). In line with these results, the cowpox virus serpin CrmA, a potent inhibitor of ICE (20) (Kₘ < 4 pm) but not of apopain (no inhibition at more than 1000 times higher concentrations (17)) did not interfere with either the PARP cleavage activity or the nuclear degradation activity of the partially purified protease at concentrations up to 0.1 μM (not shown). These results provide further evidence that CPP32/apopain is activated in Fas-mediated apoptosis.

A kinetic analysis of the extracts obtained from Fas-treated Jurkat T cells and the partially purified protease using a continuous fluorometric assay for apopain with the substrate Ac-DEVD-AMC (17) (Table I) confirmed that the proapoptotic protease from anti-Fas-treated Jurkat T cells was apopain. The catalytic and inhibitor constants obtained for the isolated protease were virtually identical with those of purified apopain (Table I). A quantitative analysis of the samples of the time course using a fluorometric assay indicated that significant active apopain was generated within 15 min following anti-Fas treatment (Table I).

To test the ability of the inhibitors used in this study to block Fas-induced apoptosis in whole cells, we preincubated Jurkat T cells with the ICE inhibitor Ac-YVAD-CHO, the apopain inhibitor Ac-DEVD-CHO, and, as a further control, the corresponding peptide lacking the aldehyde group (Ac-DEVD-COOH) which does not affect apopain activity (17). Ac-DEVD-CHO at a concentration of 120 μM was found to inhibit Fas-induced apoptosis in Jurkat T cells, whereas Ac-YVAD-CHO and Ac-DEVD-COOH were without effect (Fig. 3). Since the cells showed a viability of >95% during the entire experiment (as assessed by trypan blue exclusion; not shown), the inhibitory effect of Ac-DEVD-CHO was not due to cytotoxic effects, but rather to the inhibition of apopain. Trypan blue uptake is associated with postapoptotic necrosis and a late event in this experimental model occurring well after oligonucleosomal DNA fragmentation (>3 h) (16).

In summary, our results provide strong evidence that apopain (CPP32) is activated in Fas-mediated apoptosis. Since apopain is a key enzyme initiating apoptotic events in other systems as well (17, 18), pharmacological modulation of this protease may be of clinical importance in many diseases involving inappropriate apoptosis. Finally, we cannot exclude that additional ICE-like proteases are activated in Fas-mediated apoptosis. Although we failed to demonstrate any activation of ICE itself in anti-Fas-treated Jurkat cells, previous work has suggested that ICE may be involved in Fas-mediated apoptosis in other cell types (9, 10). Thus, further studies are required to resolve this discrepancy.

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REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Martin, S. J., Green, D. R., and Cotter, T. G. (1994) Trends Biochem. Sci. 19, 26–30
3. Barr, P. J., and Tomei, L. D. (1994) Bio/Technology 12, 487–493
4. Carson, D. A., and Ribeiro, J. M. (1994) Lancet 343, 1251–1254
5. Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991) Annu. Rev. Cell Biol. 7, 663–698
6. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjugo, G., Palayoa, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumber, R. A., Schmidt, J. A., and Tocci, M. J. (1993) Nature 365, 768–774
7. Cerecchi, D. P., Kostosky, C. J., Mosley, B., Nelson, N. E., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Hueber, K., and Black, R. A. (1992) Science 256, 97–100
8. Yuan, J., Shahaum, S., Ledoux, S., Ellis, J. M., and Horvitz, J. R. (1993) Cell 75, 641–652
9. Los, M., Van de Craen, M., Penning, L. C., Schenk, H., Westendorp, M., Baeverfr, P. A., Drige, W., and Schuiize-Osthoff, K. (1999) Nature 375, 81–83
10. Enari, M., Hug, H., and Nagata, S. (1995) Nature 375, 78–81
11. Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S.-S., and Flavell, R. A. (1995) Science 267, 2000–2003
12. Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) J. Cell Biol. 123, 7–22
13. Chow, S. C., Weis, M., Kass, G. E. N., Holmström, T. H., Eriksson, J. E., and Orrenius, S. (1995) FEBS Lett. 364, 134–138

Fig. 2. a, inhibition of the PARP cleavage activity of the partially purified protease from anti-Fas-treated Jurkat T cells by peptide aldehyde. [35S]PARP was incubated with purified apopain/CPP32 or the partially purified protease in the absence or presence of Ac-YVAD-CHO, a specific inhibitor for ICE, or Ac-DEVD-CHO, a specific inhibitor for apopain/CPP32 as described (17). b and c, Ac-DEVD-CHO inhibits apoptotic changes in vitro at nanomolar concentrations. Thymocyte nuclei were incubated with the partially purified protease from Jurkat T cells undergoing Fas-induced apoptosis in the presence or absence of Ac-YVAD-CHO or Ac-DEVD-CHO and DNA degradation into high molecular weight (b) and oligonucleosomal fragments (c) was analyzed as described under “Experimental Procedures.”

Fig. 3. Inhibition of Fas-mediated apoptosis in intact Jurkat T cells by the specific tetrapeptide aldehyde inhibitor for apopain, Ac-DEVD-CHO. Jurkat T cells (10 × 10⁶/ml) preincubated at 37 °C for 1 h with 120 μM Ac-YVAD-CHO, 120 μM Ac-DEVD-CHO, or 120 μM Ac-DEVD-COOH before incubation with anti-Fas antibody (0.75 μg/ml) for an additional 2 h were isolated by centrifugation, washed, and analyzed for DNA fragmentation as described under “Experimental Procedures.”
14. Schlegel, J., Peters, I., and Orrenius, S. (1995) FEBS Lett. 364, 139–142
15. Wilson, K. P., Black, J.-A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Nava, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 270–275
16. Weis, M., Schlegel, J., Kass, G. E. N., Holmströmn, T. H., Peters, I., Eriksson, J., Orrenius, S., and Chow, S. C. (1995) Exp. Cell Res. 219, 699–708
17. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. R., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995) Nature 376, 37–43
18. Lazebnik, Y. A., Kaufmann, S. H., Denoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347
19. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) J. Biol. Chem. 269, 30761–30764
20. Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. (1992) Cell 69, 597–604

CPP32/ Apopain in Fas-mediated Apoptosis
CPP32/Apopain Is a Key Interleukin 1 Converting Enzyme-like Protease Involved in Fas-mediated Apoptosis

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