Evidence for CPP32 Activation in the Absence of Apoptosis during T Lymphocyte Stimulation*

(Received for publication, March 7, 1997)

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Cysteine proteases of the interleukin-1β-converting enzyme family have been implicated in the effector process of apoptosis in several systems. Among these, CPP32 has been shown to be processed to active enzyme at the onset of apoptosis. Here, we show that CPP32 precursor is cleaved into its active form during phytohaemagglutinin A activation of T lymphocytes. Maximal processing is observed between day 3 and day 4 following addition of mitogen and is a transient process. Precursor cleavage is associated with the appearance of a CPP32-like enzymatic activity in cell lysates. At this time in the culture, almost no apoptotic cell and no dead cell can be detected, and T lymphocytes are actively proliferating. CPP32 processing also occurs when lymphocytes are stimulated through an allogeneic primary mixed lymphocyte reaction. Our results suggest that proteolytic activation of CPP32 could be a physiological step during T lymphocyte activation. In addition, these data indicate that CPP32 activation can occur independently of programmed cell death in T lymphocytes.

Recent studies have identified a new family of proteases, designated “caspase family” to reflect the fact that they are cysteine proteases and cleave at the C terminus of aspartic acid residues (1). The prototype of this family (caspase-1) is the interleukin-1β-converting enzyme (ICE) that was originally defined as a cytosolic protease that cleaves interleukin-1β (IL-1β) precursor into its active form (2, 3). The family now includes nine additional mammalian proteins (1): caspase-2 (NEDD-2, ICH-1), caspase-3 (CPP32, Apopain, Yama), caspase-4 (TX, ICEα-II, ICH-2), caspase-5 (ICEα-III, TY), caspase-6 (Mch2), caspase-7 (Mch3, ICE-LAP3, CMH-1), caspase-8 (FLICE, MACH, Mch5), caspase-9 (Mch6, ICE-LAP6), and caspase-10 (Mch4).

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The abbreviations used are: ICE, interleukin-1β-converting enzyme; AMC, amino-4-methylcoumarin; DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aldehyde; caspase, cysteine protease; IL, interleukin; rIL, recombinant IL; MGG, May-Grünwald-Giemsa; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin A; PMA, phorbol myristate acetate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; mAb, monoclonal antibody.

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EXPERIMENTAL PROCEDURES

Activation of PBMC

Human PBMC were isolated through Ficoll-Paque density gradient centrifugation from buffy coats of healthy donors. They were stored frozen and thawed before the assay. PBMC were seeded at 0.5 × 10^6 cells/ml in supplemented Dulbecco’s modified Eagle’s medium containing 8% (v/v) human AB serum and were stimulated with 1 µg/ml PHA for 3 days. Cells were diluted in fresh medium on day 4 following PHA activation. For long-term culture, rIL-2 (5 ng/ml) was added on day 8 and every 3 days.

Allogeneic primary mixed lymphocyte reaction was carried out by mixing PBMC from two heterologous individuals. After 5 and 6 days of culture, Ficoll-Paque density gradient centrifugation was performed to eliminate nonactivated dying cells from blasts. 100% viability in blast lymphocytes was checked by trypan blue dye exclusion test.
Activation of Jurkat Cells

Jurkat cells were maintained in supplemented RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum. Jurkat cells were activated with PHA (1 μg/ml) or with a combination of PHA and PMA (1 μg/ml and 1 ng/ml, respectively) and incubated for 24 or 48 h.

Cell Lysis, Western Blotting, and Immunodetection

As described previously (18), cell pellets were resuspended in ice-cold hypotonic lysis buffer (20 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1 mM EDTA, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, leupeptin, and pepstatin, and 1 mM N-ethylmaleimide). Lysates from approximately 2 × 10^6 cells were run on SDS-PAGE and proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon). The blots were probed with either anti-CPP32 or anti-ICH1, mouse mAb (Transduction Laboratories) and with a horseradish peroxidase-conjugated goat anti-mouse IgG. Proteins were visualized using the enhanced chemiluminescence detection system (ECL™, Amersham Corp.).

Cell Surface Immunofluorescence Staining and Cytocfluorimetric Analysis

The cell surface immunofluorescence assays were performed according to conventional procedures. Briefly, cells were incubated for 30 min at 4 °C with a predetermined concentration of fluorescein isothiocyanate-conjugated Ab (Immunotech): anti-CD3 or anti-CD25 mAb or irrelevant control Ab. Samples were run on a Coulter ELITE flow cytometer, and 10,000 events were analyzed.

CPP32 Activity Assay

10^6 cells were lysed in 200 μl of ice-cold hypotonic buffer (20 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1 mM EDTA, and 10 μg/ml trypsin inhibitor). Cell lysis was fulfilled by two cycles of freezing at −80 °C and thawing at 4 °C, to prevent nonspecific cleavage of proteins. The homogenates were clarified by a 10-min centrifugation at 13,000 rpm. Supernatants were collected and diluted in 50 mM Tris-HCl, pH 7.5, 0.1% CHAPS, and 10 mM dithiothreitol for enzyme assays. Enzymatic reactions were carried out in triplicates in a total volume of 100 μl containing lysate from 12.5 × 10^6 cells, 50 μM DEVD-AMC (Bachem), and various concentrations of DEVD-CHO (Bachem) or YVAD-CEO (Rousell Uclaf). Release of AMC was monitored during 1 h at 37 °C, on a microplate fluorimeter (Fluostar), with excitation and emission wavelength of 390 and 460 nm, respectively.

Detection of Apoptosis

Morphological Examination—Cells were cytopun and stained with methylene blue and eosin by May-Grünwald-Giemsa (MGG) procedure. Nuclear morphology was observed under light microscopy.

Detection of Apoptosis by Cell Cycle Analysis—Cell cycle analysis was performed as described previously (14). Briefly, PHA-activated cells were fixed by a 15-min incubation in ice-cold phosphate-buffered saline, 1% paraformaldehyde, followed by a 45-min incubation in ethanol 70% at −20 °C. Cells were then incubated 15 min in the dark with propidium iodide (50 μg/ml) and RNase A (50 μg/ml). Samples were analyzed by flow cytometry (Coulter ELITE), and apoptotic cells were discriminated from their healthy counterparts as a distinct peak below the G1/G0 peak of the cell cycle. Percentage of apoptotic cells was defined as follows: cells in sub-G1/G0 region/cells in sub-G1/G0 region + cells in cell cycle × 100.

RESULTS AND DISCUSSION

CPP32 Expression in PHA-stimulated PBMC—PHA-stimulated PBMC were used as a model of cells undergoing activation and proliferation. Cells were harvested after various periods of culture and cell lysates were analyzed by immunoblotting with a CPP32-specific mAb. Fig. 1A shows the results obtained at different time up to day 8 following addition of PHA. The 32-kDa precursor of CPP32 is constitutively expressed in unstimulated PBMC, and it is the only form detected in these cells. In contrast, in PHA-stimulated cells, the amount of CPP32 precursor was found to decrease dramatically by day 3. In parallel, new signals appeared at about 17 and 20 kDa, most probably corresponding to the 17 large subunit of CPP32 and the 20 kDa product constituted of p17 plus the precursor propiece. The mAb used in these experiments is directed at the p20 subunit and therefore does not detect the p17 subunit. In the experiment shown in Fig. 1, maximal effects were observed on days 3 and 4, with the pool of CPP32 precursor being nearly totally processed. In other kinetic experiments with PBMC from various individuals, maximal CPP32 cleavage varied from day 3 to day 5. CPP32 precursor cleavage seemed to be transient in the culture and by day 8, only minimal processing was observed (Fig. 1A). This was confirmed by further experiments where PBMC were activated during prolonged periods by adding rIL-2 to the culture every 3 days from day 8. Under these conditions, almost no CPP32 cleavage product could be detected by days 15 and 22 of culture (Fig. 1B).

We also studied the expression of ICH1, another member of the ICE family protease, whose expression was previously shown to remain unchanged in apoptotic cells (14). We found that ICH1 precursor expression pattern was not modified during PHA stimulation or after prolonged culture with rIL-2 (Fig. 1A and B). Similarly, ICE and TX precursor expression were found unchanged during PHA stimulation (data not shown).

These results therefore indicate that during PBMC stimulation with a T lymphocyte-specific mitogen, processing of CPP32 into its subunits is observed in cells cultured for 3–5 days. Upon further culture, the cells return to their initial CPP32 expression pattern by days 10–15. This reversion to initial conditions could be explained by new synthesis of the CPP32 precursor together with degradation of the p20 subunits in the cells or by the progressive renewal of cells in the culture.

CPP32 Enzymatic Activity in Activated Cells—To verify that CPP32 precursor cleavage was associated with activation of the enzyme, we measured CPP32 enzymatic activity in the cell lysates at day 0 and after 4 days of culture with PHA. Enzymatic activity was monitored during 1 h by measuring the cleavage of the fluorogenic substrate DEVD-AMC, which was shown to be one of the preferred substrates for CPP32, with a Km value of 9.7 μM (7). As shown in Fig. 2A, there was no cleavage of DEVD-AMC by lysates of nonactivated PBMC. In contrast, significant DEVD-AMC cleavage activity was observed in lysates from cells cultivated with PHA for 4 days (Fig.
DEVD-AMC cleavage can be inhibited by the tetrapeptide aldehyde DEVD-CHO, which is a potent competitive inhibitor of CPP32 enzyme with a $K_i < 1 \text{ nM}$ (7). In our assay, 1 and 0.1 µM of DEVD-CHO totally blocked AMC liberation, and about 50% of inhibition was obtained with the dose of 0.01 µM (Fig. 2B). The ICE inhibitor YVAD-CHO, which is a weak inhibitor of CPP32 with a $K_i$ of 12 µM (7), was also tested. At doses up to 1 µM, it was without effect on DEVD-AMC cleavage by PHA-stimulated cell lytsates (Fig. 2B). Taken together, these data confirm the presence of a CPP32-like enzymatic activity in PHA-activated cell lysates. An immunoblotting was also performed with the same lysates, to verify that CPP32 processing had occurred in this experiment (data not shown). Therefore, we can conclude from these experiments that CPP32 process-

![Figure 2](image)

**FIG. 2.** CPP32-like activity in lysates from PHA-stimulated PBMC. Lysates from 12.5 × 10^6 unstimulated PBMC or day 4 PHA-activated PBMC were incubated with 50 µM of DEVD-AMC substrate in a 100-µl total volume. Cleavage of fluorogenic DEVD-AMC was monitored continuously for 1 h at 37 °C (1000 fluorescence units represent 4.25 pmol of AMC). A, kinetic of DEVD-AMC cleavage. Rate of cleavage was 0.3 mmol/min for the unstimulated PBMC lysate (full squares) and 34.4 mmol/min for the activated PBMC lysate (open circles). B, effect of DEVD-CHO and YVAD-CHO on DEVD-AMC cleavage. DEVD-CHO (triangles) or YVAD-CHO (circles) inhibitors were added at various concentrations (10, 100, or 1000 nM) to theoretical reaction mixtures containing PHA-activated cell lysates. Cleavage activity is reported relative to the activity of the same lysate in the absence of inhibitor.

![Characterization of PHA-activated Cells after 4 and 5 Days in Culture](image)

**FIG. 3.** Characterization of PHA-activated lymphocytes after 4 and 5 days in culture. PBMC were incubated in the presence of PHA and harvested after 4 and 5 days in culture. A, immunofluorescence analysis. Cytofluorimetric analysis was performed after cell surface staining with anti-CD3, anti-CD25, or negative control mAb. The x axis shows fluorescence intensity on a logarithmic scale, and the y axis represents the relative frequency of cells. Untreated PBMC (day 0) were analyzed in parallel with day 4 and day 5 activated cells. B, morphological examination. Cells were cytocentrifuged and stained by MGG procedure. Microscopic examination was performed with an original magnification of ×400. C, quantitative measurements of cell numbers, dead cells, and apoptotic cells. Cells counts and viability were assessed by trypan blue exclusion test. Apoptosis was scored following cell permeabilization, staining with propidium iodide, and analysis by flow cytometry. Percentage of apoptotic cells was calculated as indicated under “Experimental Procedures.” D, immunoblotting of CPP32 protein. Cell lysates were subjected to 12% SDS-PAGE, blotted, and probed with anti-CPP32 or anti-ICH1 mAb as indicated. Numbers on the left show the molecular size of standards in kilodaltons.
proliferate with viable cell counts growing from 0.67 \times 10^6 cells/ml on day 4 to 1.05 \times 10^6 cells/ml on day 5 (Fig. 3C). As a control, Fig. 3D shows that in the same experiment the majority of the cells were undergoing CPP32 processing, with a maximum effect by day 4. Therefore, in contrast with the experiments described to date where CPP32 activation is linked to programmed cell death, we observe CPP32 processing in the absence of any detectable cell death or apoptosis in the culture and at a time when cell proliferation is at its maximum.

Expression of CPP32 in Mixed Lymphocyte Reaction—Mitogenic stimulation with lectins is a very potent polyclonal signal for any detectable cell death or apoptosis in the culture and at a maximum effect by day 4. Therefore, in contrast with the experiments of the cells were undergoing CPP32 processing, with a maximum of culture. Cell lysates were subjected to 12\% SDS-PAGE, blotted, and probed with anti-CPP32 or anti-ICH1 mAb as indicated. Numbers on the right show the molecular size of standards in kilodaltons. ICH1 precursor migration on a separate gel was compatible with its theoretical molecular mass (48 kDa).

In conclusion, we have observed that CPP32 activation through precursor processing can occur in situations where the cells are activated but are not undergoing apoptosis. This processing is associated with the appearance of an intracellular CPP32-like enzymatic activity and therefore represents true activation of the enzyme. Finally, this phenomenon appears to be transient, since cells cultured for prolonged periods or long term cell lines fail to show CPP32 processing. Further studies will be required to determine whether this only occurs in T lymphocytes or is seen in other cell types as well. In addition, determination of the function and the substrates of CPP32 in cells not undergoing apoptosis will be of great interest to understand the role of this protease and its relationship to cell physiology and cell death.

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FIG. 4. CPP32 processing during mixed lymphocyte reaction. PBMC from two heterologous individuals were mixed and incubated together. Cells were harvested after 5 or 6 days in culture, and dead cells were eliminated by centrifugation through Ficoll-Paque density gradient. Cell lysates were subjected to 12\% SDS-PAGE, blotted, and probed with anti-CPP32 or anti-ICH1 mAb as indicated. Numbers on the right show the molecular size of standards in kilodaltons. ICH1 precursor migration on a separate gel was compatible with its theoretical molecular mass (48 kDa).

FIG. 5. Absence of CPP32 processing in PHA- or PHA/PMA-activated Jurkat cell line. Jurkat cells were incubated alone or in the presence of PHA or PHA and PMA. Cells were harvested after 24 h of culture. Cell lysates were subjected to 12\% SDS-PAGE, blotted, and probed with anti-CPP32 or anti-ICH1 mAb as indicated. Numbers on the right show the molecular size of standards in kilodaltons.
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J. Biol. Chem. 1997, 272:13459-13462. doi: 10.1074/jbc.272.21.13459

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