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Granzyme Release and Caspase Activation in Activated Human T-Lymphocytes*

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Recently it has been reported that caspase-3 activation occurs in stimulated T-lymphocytes without associated apoptosis (Miossec, C., Dutilleul, V., Fassy, F., and Diu-Hercend, A. (1997) *J. Biol. Chem.* 272, 13459–13462). To explore this phenomenon, human peripheral blood lymphocytes (PBLs) were stimulated with mitogenic lectins or anti-CD3 antibody, and the proteolytic processing of different caspases and caspase substrates was analyzed by immunoblotting. Proteolytic processing of caspases-3 and -7 and the caspase substrates poly(ADP-ribose) polymerase, GDP dissociation inhibitor, and PKCδ was observed when PBLs were activated *in vitro*, and lysates were prepared using RIPA buffer which contains 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. In contrast, when a lysis buffer containing 2% SDS was used, the caspases remained in their zymogen proforms, and no proteolytic processing of caspase substrates was detected. Moreover, in experiments using intact cells and a cell-permeable fluorigenic caspase substrate, no caspase activity was observed in activated T-cells, whereas it was clearly detected when PBLs were treated with the apoptosis-inducing anticancer drug etoposide. Since the granzyme B is a direct activator of caspase-3 and its expression is induced following T-cell activation, we tested the effects of anti-GraB, an engineered serpin that specifically inhibits GraB. When the activated T-lymphocytes were lysed in RIPA buffer containing anti-GraB, no proteolytic processing or activation of caspase-3 was observed, strongly suggesting that release of GraB or similar proteases from their storage sites in cytotoxic granules during the lysis procedure is responsible for caspase activation. These findings demonstrate that T-cells do not process caspases upon activation and caution about the method of cell lysis used when studying granzyme-expressing cells.

Caspases are a family of cysteine proteases related to the mammalian interleukin-1β (IL-1β)[1] converting enzyme (caspase-1) that processes pro-IL-1β and to the nematode CED-3 protease that plays a central role in programmed cell death in that organism (1–4). Diverse stimuli that cause apoptosis result in activation of these cysteine proteases, which have specificity for aspartic acid in the P1 position of substrates. Accordingly, specific peptidyl inhibitors of caspases are potent inhibitors of apoptosis, thus implying that caspases play an essential role as either initiators or effectors of apoptotic cell death pathways. Experiments involving caspase gene disruption in mice also support the idea of a critical role for caspases in apoptosis (5, 6). Several cellular substrates of caspases have been identified, including certain protein kinases, enzymes involved in DNA repair, and proteins that regulate cytoskeleton integrity (7–25). It is believed that the combined cleavage of multiple substrates by caspases directly contributes to the demise of the cell.

Nevertheless, caspase involvement in cellular processes other than apoptosis has also been documented. In this regard, the caspase-1 subfamily (which includes caspases-1, -4, and -5) is primarily involved in the processing of pro-IL-1β (26, 27) and pro-interferon-γ inducing Factor (28, 29). Both, IL-1β and interferon-γ are pleiotropic cytokines that modulate several functions of immune cells (28, 29).

In contrast to the caspase-1 subfamily which is involved in cytokine pro-protein processing, caspase-3 and the other CED-3-like members of the caspase family have uniformly been associated with apoptosis, and no other function for these proteases is known. Recently, however, Miossec *et al.* (30) reported that caspase-3 activation might be involved in regulating proliferation and lymphocyte activation events in T-cells, in the absence of associated apoptosis. In this report, we demonstrate that caspase-3-like proteases are not normally activated during T-cell stimulation, and therefore they are unlikely to play a role in lymphocyte activation or proliferation. Moreover, our findings suggest that post-lysis release from intracellular granules of GraB or similar serine proteases with specificity for aspartic acid can lead to artifactual proteolytic processing and activation of caspases.

EXPERIMENTAL PROCEDURES

Materials—The caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) and acetyl-Asp-Glu-Val-Asp-fmk (Ac-DEVD-fmk) were purchased from Enzyme Systems Products (Livermore, CA) and dissolved in Me_2SO. The preparation and purification of the active caspase-3 and the specific granzyme B inhibitor anti-GraB have been described elsewhere (31–33).

Isolation and Activation of PBLs—PBLs were isolated from heparinized venous blood from human volunteers by Ficoll-histopaque (Sigma) centrifugation. PBLs were left unstimulated or treated at the onset with 5 μg/ml phytohemagglutinin (Difco). After 2 days, cells were washed and incubated thereafter with 50 units/ml IL-2 (Genzyme, Boston, MA). In some experiments, PBLs were activated with 2.5 μg/ml anti-CD3 mAb OKT3 (kindly provided by Orthodiagnostics, Inc. Stamford, CT) or with 5 μg/ml concanavalin A (Sigma).

Cell Extracts—Cell extracts were made using two different protocols. The first one involved lysis of $10–50 \times 10^6$ cells in 50–200 μl of either RIPA buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) or Nonidet P-40 lysis

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[1] The abbreviations used are: IL, interleukin; PBL, peripheral blood lymphocytes; PARP, poly(ADP-ribose) polymerase; GDI, GTP-dissociation inhibitor; PKC, protein kinase C; AFC, 7-amino-4-trifluoromethylcoumarin; fmk, fluoromethylketone; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GraB, granzyme B; Me_2SO, dimethyl sulfoxide; Z, benzyloxycarbonyl.

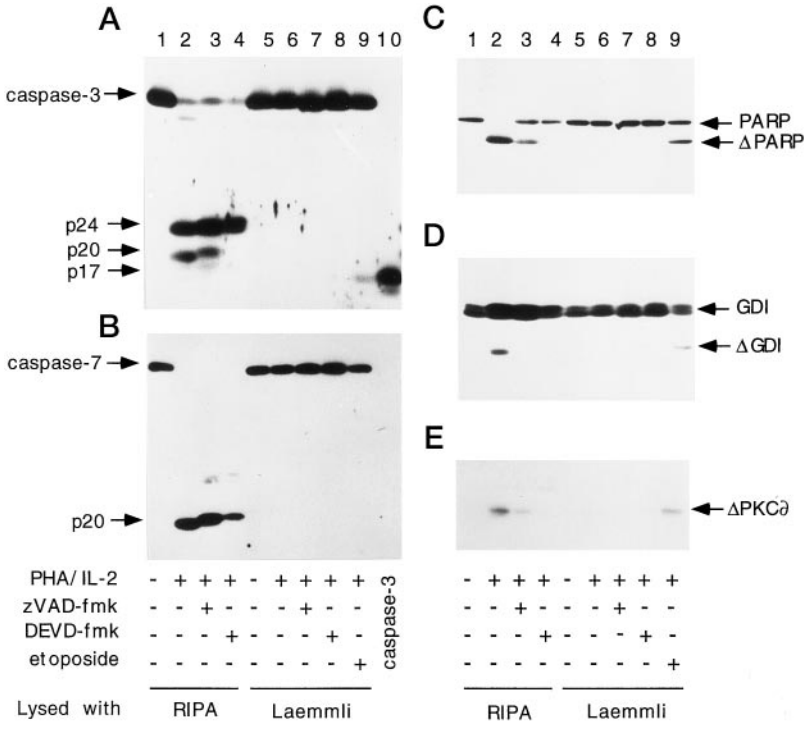


FIG. 1. **Effect of different buffers on the activation of caspases in stimulated human PBLs.** PBLs were either untreated (*lanes 1* and *5*) or activated with phytohemagglutinin and IL-2 for 7 days (*lanes 2–4* and *6–9*), and then cells were lysed with RIPA (*lanes 1–4*) or Laemmli buffer (*lanes 5–9*). Where indicated, cells were cultured in the presence of the caspase inhibitors Z-VAD-fmk (*lanes 3* and *7*) or Ac-DEVD-fmk (*lanes 4* and *8*) at 25 μM. In some cases, activated PBLs were cultured with etoposide (5 μg/ml) for 6 h (*lane 9*) prior to lysis. Cell lysates (50 μg) were subjected to SDS-polyacrylamide gel electrophoresis (12%) and immunoblotting, using antibodies specific for caspase-3 (*B*), caspase-7 (*C*), PARP (*D*), D4-GDI (*E*), or the cleaved product of PKCδ (*F*). In *lane 10*, 50 ng of purified recombinant caspase-3 which consists of p17 and p12 subunits was loaded directly into the gel. The positions of the various proteins and their proteolytic fragments are indicated.

buffer (10 mM Hepes, pH 7.4, 142 mM KCl, 1 mM EGTA, 1 mM dithiothreitol, and 0.2% Nonidet P-40) on ice for 2 min, followed by removal of DNA and cell debris by centrifugation at 16,000 × *g* for 5 min at 4 °C. The resulting supernatants were collected and frozen at −80 °C or used immediately. The second protocol involved lysis with modified Laemmli buffer (60 mM Tris, pH 6.8, 10% glycerol, and 2% SDS, without β-mercaptoethanol and bromphenol blue). Cell disruption was completed by sonication for 30 s on ice, followed by centrifugation as above, and the supernatants were frozen at −80 °C or used immediately. Protein concentration was quantified using the bicinchoninic acid (BCA) method (Pierce).

Immunoblotting—Immunoblot analyses were performed as described in detail elsewhere, using the multiple antigen detection immunoblotting method (34). Briefly, lysates were normalized for total protein content (50 μg per lane) and subjected to SDS-polyacrylamide gel electrophoresis (12% gel), followed by transfer to nitrocellulose filters (0.4 μm). The primary antibodies employed were directed against caspase-3 (35), caspase-7 (kindly provided by Dr. Visha Dixit, Genentech, San Francisco, CA) (36), PARP (kindly provided by Dr. Guy Poirier, Universite Laval, Quebec, Canada) (37), D4-GDI (kindly provided by Dr. Gary M. Bokoch, Scripps Research Institute, La Jolla, CA) (19), and PKCδ (Santa Cruz Biotechnology).

Detection of Active Caspases in Intact Cells—The cell-permeable fluorigenic substrate (PhiPhilux-G_6D_2) was used to monitor caspase activity in intact cells, according to the manufacturer's recommendations (OncoImmunin, Inc, Kensington, MD). Briefly, cells were collected and washed twice in PBS. Then, 10^6 cells were resuspended in 50 μl of substrate solution and supplemented with 5 μl of fetal calf serum. Cells were incubated for 1 h at 37 °C in a water bath in the dark. After incubation, cells were washed once and the fluorescence emission was determined using the FL-2 channel of a Becton Dickinson FACSort flow cytometer.

In Vitro Caspase Activity Assay—Caspase-3-like protease activity was measured at 37 °C using a spectrofluorometric plate reader (Perkin-Elmer, LS50B) in the kinetic mode with excitation and emission wavelengths of 400 and 505 nm, respectively. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate Z-DEVD-AFC (Enzyme Systems Products, Livermore, CA). Lysates (5–10 μg of total protein) were mixed with 100 μl of caspase buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, and 5 mM dithiothreitol), and reactions were initiated by addition of 100 μM of Z-DEVD-AFC.

RESULTS AND DISCUSSION

To assess the putative role of caspases in T-cell activation events, human PBLs were either cultured for up to 7 days in medium without stimulants or were activated *in vitro* using either phytohemagglutinin or concanavalin A in combination with IL-2 or by anti-CD3 monoclonal antibody. These activated cells were actively proliferating, and >99% were non-apoptotic as determined by microscopic evaluation of 4−6-diamidino-2-phenylindole-stained cells and by flow cytometric DNA content analysis (not shown). T-cells were recovered from cultures at different times and lysed in RIPA buffer, and the proteolytic processing of caspases and caspase substrates was evaluated by immunoblotting.

In lysates derived from unstimulated PBLs, caspases and caspase substrates were consistently present in their unprocessed forms (Fig. 1, *A–E, lane 1*). In contrast, in all experiments where PBLs were activated *in vitro* and cells were lysed in RIPA buffer (n = 6), marked reductions in the ~36-kDa proform of caspase-3 were found, and two processed forms of ~24 and ~20 kDa appeared (Fig. 1*A, lane 2*), similar to results described by Miossec *et al.* (30). The p24 form of caspase-3 has been previously attributed to the cleavage at Asp-175, which produces an ~24-kDa large subunit and ~12-kDa small subunit, the latter of which is not detected by the antibody employed here (35). Further processing of the large subunit usually at either Asp-9 or Asp-28 to yield p20 or p17 large subunits, respectively, has also been described (38). The p24 and p20 processed forms of caspase-3 seen in activated PBL lysates appear to represent active proteases, based on experiments where an irreversible biotinylated caspase-3 substrate, biotin-VAD-fmk (39), was incubated with these lysates, and both the p24 and p20 bands were subsequently shown to bind avidin-conjugated alkaline phosphatase (not shown).

Similar results were obtained when caspase-7 was analyzed. As shown in Fig. 1*B*, unstimulated PBLs contained exclusively the unprocessed ~38-kDa zymogen form of caspase-7, whereas only the ~20-kDa processed large subunit of this protease was detected in RIPA lysates from activated PBLs (*lanes 1* and *2*). In addition, the caspase substrates PARP, D4-GDI, and PKCδ (10, 16, 19) were found to be at least partially cleaved in lysates derived from activated but not unstimulated PBLs (Fig. 1, *C–E, lanes 1* and *2*). This same pattern of caspase and caspase substrate cleavage was observed for PBLs that had been stim-

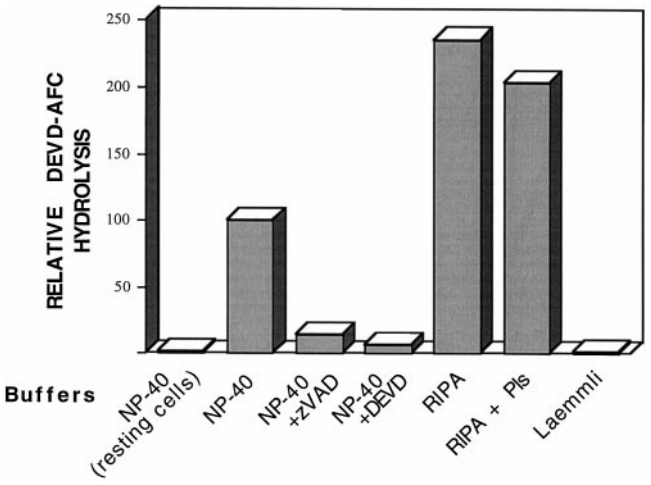


FIG. 2. **RIPA and Nonidet P-40 lysates from activated PBLs contain caspase-3-like activity.** Resting (first sample) or activated (all others) PBLs were lysed in Nonidet P-40 (*NP-40*), RIPA, or Laemmli-based buffers as indicated. Lysates (5 μg of total protein) were incubated in 100 μl of caspase buffer in the presence of 100 μM z-DEVD-AFC. The rate of substrate hydrolysis was calculated after allowing reactions to reach steady state and is shown as percent activation, considering the activity obtained in a isotonic buffer containing 0.2% Nonidet P-40 as 100%. The concentrations of Z-VAD-fmk and Ac-DEVD-fmk used were 25 μM. The serine protease inhibitors (*PIs*) were phenylmethylsulfonyl fluoride (1 mM), leupeptin (50 μg/ml), benzamidine (1 mM), pepstatin (0.7 μg/ml), and aprotinin (20 μg/ml).

ulated in culture with mitogenic lectins and IL-2 for 3–14 days and lysed in RIPA (not shown).

Surprisingly, culturing PBLs with 25 μM cell-permeable broad specificity caspase inhibitor Z-VAD-fmk or the relatively caspase-3/caspase-7-selective inhibitor Ac-DEVD-fmk failed to prevent the proteolytic processing of the pro-forms of caspases-3 and -7, implying that a Z-VAD and Ac-DEVD-resistant protease was responsible (Fig. 1, *A* and *B*, *lanes 3* and *4*). In contrast, these same peptidyl inhibitors of caspases were effective blockers of caspase processing in other types of cells when used at the same or even 10-fold lower concentrations (not shown). The Z-VAD-fmk and Ac-DEVD-fmk reagents, however, did at least partially inhibit proteolytic processing of PARP, D4-GDI, and PKCδ, implying that caspases were responsible for these proteolytic events and confirming that the peptidyl inhibitors were indeed active (Fig. 1, *C–E, lanes 3* and *4*).

Lysates derived from activated but not resting PBLs that had been made with RIPA buffer or with an isotonic buffer containing 0.2% Nonidet P-40 as the only detergent contained active caspases, as determined by *in vitro* measurements of Z-DEVD-AFC hydrolysis (Fig. 2). In contrast, lysates derived from PBLs that had been activated in culture in the presence of Z-VAD-fmk or Ac-DEVD-fmk contained far less Z-DEVD-AFC hydrolyzing enzymes. This result implies that while processing of caspase-3 and caspase-7 had occurred in these lysates (Fig. 1), the activated caspases were effectively inhibited by Z-VAD-fmk and DEVD-fmk. Addition to PBL lysates of a combination of serine protease inhibitors, including aprotinin, phenylmethylsulfonyl fluoride, benzamidine, leupeptin, and pepstatin, failed to suppress the caspase activity that was present in these extracts (Fig. 2), thus confirming the caspase specificity of the proteolytic activity detected by this Z-DEVD-AFC hydrolysis assay.

To explore further the mechanisms responsible for the proteolytic processing and activation of caspases observed in these experiments with activated T-cells, PBLs were lysed in a modified Laemmli solution that contained 2% SDS instead of RIPA or Nonidet P-40 lysis buffer. When the same preparations of activated T-cells were lysed in this 2% SDS solution, no proteolytic processing of caspases-3 or -7 was found by immunoblot analysis (Fig. 1, *A* and *B*; *lane 6*). Moreover, no cleavage of PARP, D4-GDI, or PKCδ was detected under these conditions (Fig. 1, *C–E, lane 6*). Although the high concentrations of SDS in these lysates may have precluded effective assay of these proteases, no elevations in Z-DEVD-AFC hydrolytic activity were detected (Fig. 2).

As a control to verify that lysis of cells in Laemmli buffer did not preclude recovery of processed caspases or caspase substrates, activated PBL were treated with the anticancer drug etoposide which induced approximately half the cells to undergo apoptosis under these conditions, then lysed with Laemmli buffer, and analyzed by immunoblotting. As shown in Fig. 1*A* (*lane 9*), etoposide-treated PBLs contained the ~17-kDa processed form of caspase-3 corresponding to the fully processed large subunit of the enzyme (38). This 17-kDa caspase-3 band co-migrated with the purified recombinant p17 large subunit of caspase-3 produced in bacteria (*A, lane 10*). Although etoposide-treated PBLs contained little processed caspase-7, cleavaged PARP, D4-GDI, and PKCδ were readily detected in these lysates (Figs. 1, *C–E, lane 9*). Altogether, these results strongly suggest that activation of the caspases and subsequent proteolysis of their substrates in activated T-lymphocytes occur after mechanical disruption of the cells in RIPA buffer.

A cell-permeable fluorigenic caspase substrate PhiPhilux-G_6D_2 was used to evaluate further whether caspases become activated in intact PBLs after stimulation with mitogenic lectins and IL-2. Very few cells produced fluorescence emissions above background in cultures of activated PBLs (Fig. 3*A*), whereas over half the etoposide-treated PBLs apparently cleaved the PhiPhilux-G_6D_2 substrate (Fig. 2*B*). To confirm that the cleavage of the fluorigenic substrate PhiPhilux-G_6D_2 seen in etoposide-treated T-cells was indeed caspase-dependent, these cells were pretreated with either Z-VAD-fmk or Ac-DEVD-fmk prior to exposure to PhiPhilux-G_6D_2, demonstrating nearly complete suppression of the fluorescence emission (Fig. 3, *C* and *D*). These results therefore demonstrate that while caspase-3-like proteases are activated in T-cells treated with the apoptosis-inducing anticancer drug etoposide, these proteases evidently do not become activated within T-cells after stimulation with mitogens and lymphokines under conditions where apoptosis does not occur. In agreement with these observations, no effect of the caspase inhibitors Z-VAD-fmk or Ac-DEVD-fmk was seen on proliferation rates or on expression of T-cell surface activation markers (CD25 and CD71) (not shown).

Comparisons of caspase processing in lysates prepared with RIPA *versus* Laemmli buffer using a variety of non-T-cell lines suggested that post-lysis activation of these proteases may be relatively unique to activated PBLs (not shown). We hypothesized, therefore, that one possible explanation for the post-lysis activation of caspases seen with activated PBLs might be the presence of GraB in the cytolytic subpopulation of T-lymphocytes (40). GraB is stored in granules within cytolytic T-cells and secreted during cytolytic T-cell-mediated killing of target cell. GraB is a direct activator of caspase-3 (32, 41–43) and caspase-7 (36, 44, 45), as well as other caspases, including caspases-6, -9, and -10 (45–49). Furthermore, GraB expression is induced following T-cell activation (50–52).

To ascertain if the activation of caspases after PBL cell lysis is mediated by GraB, activated PBLs were lysed in RIPA buffer in the presence or absence of anti-GraB, an engineered form of the serpin antichymotrypsin which has been converted to a highly specific inhibitor of GraB (32). As shown in Fig. 4, RIPA

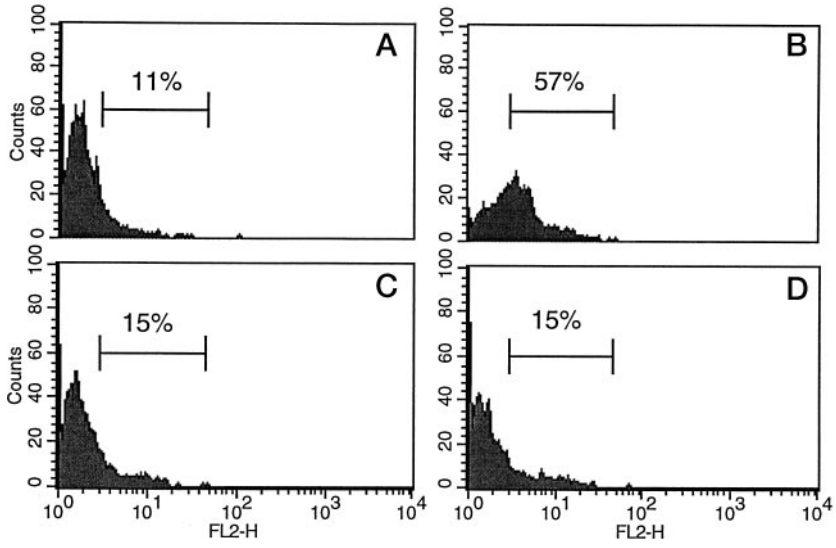
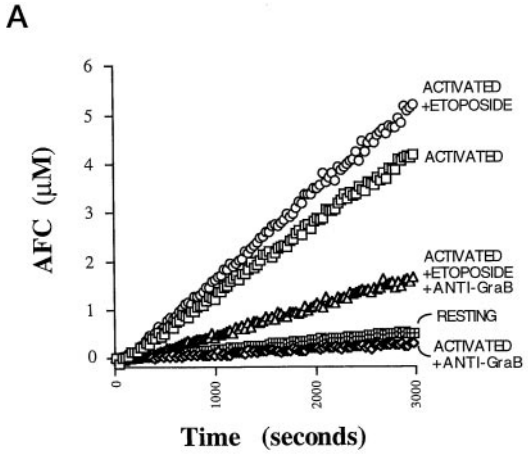


FIG. 3. **Detection of caspase activity in intact activated T-lymphocytes.** Cells were activated for 7 days with phytohemagglutinin and IL-2 and then subjected to no further treatment (*A*) or cultured with 5 μg/ml etoposide for 6 h in the absence (*B*) or in the presence of 25 μM Z-VAD-fmk (*C*) or Ac-DEVD-fmk (*D*). Cells were then incubated with a fluorigenic caspase substrate PhiPhilux-G_6D_2 for 1 h and then analyzed by fluorescence-activated cell sorter.



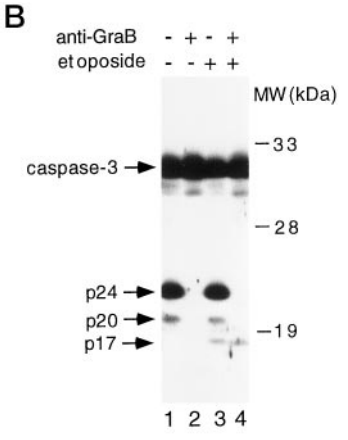


FIG. 4. **GraB accounts for post-lysis activation of caspases.** *A*, activated PBLs were subjected to either no further treatment or cultured with 5 μg/ml etoposide for 6 h. 10^6 cells were lysed in 20 μl of RIPA buffer in the presence or in the absence of 80 ng of anti-GraB. DEVD-AFC hydrolysis was measured, using 10 μg of extracts. *B*, the pattern of caspase-3 cleavage in the cell lysates described above was analyzed by SDS-polyacrylamide gel electrophoresis (12%)/immunoblotting. The positions of pro-caspase-3 and its proteolytic fragments are indicated.

lysates of activated PBLs contained DEVD-AFC cleavaging activity (Fig. 4*A*), which correlated with the presence of the p24- and p20-processed fragments of caspase-3 in extracts (Fig. 4*B, lane 1*). In contrast, when the cell lysis was performed in the presence of anti-GraB, the hydrolysis of DEVD-AFC was nearly completely abrogated, and processing of caspase-3 to the p24 and p20 fragments was inhibited (Fig. 4*B, lane 2*). Activated T-cells treated with etoposide and lysed in RIPA contained the p24 and p20 fragments of caspase-3, as well as the p17 form of the fully processed large subunit of the mature enzyme (Fig. 4*B, lane 3*). Lysis of these cells in the presence of anti-GraB blocked the formation of the p24 and p20 fragments, but the p17 form remained (Fig. 4*B, lane 4*). Accordingly, although Z-DEVD-AFC cleavage activity was partially inhibited by lysis of etoposide-treated PBLs in the presence of GraB, some protease activity remained, presumably reflecting the etoposide-induced processing of caspase-3 to the p17 mature isoform. These results suggest that anti-GraB inhibits the post-lysis processing of caspase-3 but does not interfere with the pre-lysis activation of caspases that was induced by etoposide. We cannot exclude the possibility, however, that T-cells express other granzymes besides GraB which might also possess aspartic acid specificity and which could serve as targets of the anti-GraB protein.

Overall, these results support the hypothesis that release liberation of GraB or similar proteases from cytotoxic granules during the lysis procedure results in artifactual activation of caspase-3. In this regard, lysates prepared with RIPA buffer contained more caspase activity than those generated with 0.2% Nonidet P-40, suggesting that RIPA may disrupt GraB-containing granules more efficiently. Moreover, in these experiments, GraB activated caspases-3 and -7, and the activated caspases cleaved their substrates such as PARP, D4-GDI, and PKCδ even though the lysates were kept at 0–4 °C. These observations thus highlight the importance of choosing appropriate lysis buffers for processing of cells and tissues if post-lysis activation of caspases is to be avoided. Of potential relevance, GraB-containing lymphocytes can be found in various tissues of both lymphoid and non-lymphoid origin and in both physiological and pathological situations (53–57). Therefore, the presence of GraB should be taken into consideration in studies involving regulation of apoptosis or caspase activation in such tissues. Furthermore, it cannot be excluded that other proteases might activate caspases after cell lysis. In this regard, cathepsin G and subtilisin have recently been reported to activate caspase-7 (45). In conclusion, the results presented here demonstrate that activated PBLs do not normally produce active caspase-3-like proteases under circumstances where apoptosis does not occur and warn about of the proper selection of lysis buffers for studies of GraB-containing cell populations.

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