Cytotoxic T lymphocytes (CTLs) are able to recognize and destroy target cells bearing foreign antigen using one of two distinct mechanisms: granule- or Fas-mediated cytotoxicity. The exact mechanisms involved in the induction of apoptotic cell death remain elusive; however, it seems likely that a family of cysteine proteases related to interleukin-1β-converting enzyme are involved. One family member, CPP32, has been identified as an intracellular substrate for granzyme B, a CTL-specific serine protease responsible for the early induction of target cell DNA fragmentation. Here we use cytolytic cells from granzyme B-deficient mice to confirm that cleavage and activation of CPP32 represents a non-redundant role for granzyme B and that this activation plays a role in the induction of DNA fragmentation in target cells, a signature event for apoptotic cell death. A peptide inhibitor of CPP32-like proteases confirmed the function of these enzymes in fragmentation. 51Cr release was not suppressed under these conditions, suggesting that granzyme B cleavage of CPP32 is primarily involved in the induction of DNA fragmentation and not membrane damage during CTL-induced apoptosis.

Cytotoxic T lymphocytes (CTLs) represent the body’s primary defense against tumorigenic and virus-infected cells and are responsible for organ transplant rejection and autoimmune disease (1). When the T cell receptor recognizes a foreign antigen presented in the context of the major histocompatibility complex class I, the CTL induces apoptotic cell death in the target cell through one of two distinct mechanisms: Fas-based and granule-mediated cytotoxicity. During granule-mediated killing, T cell receptor activation leads to the induction of function-related genes, including perforin and the granzymes. These are packaged into granules, which are then mobilized to the surface of the CTL that is adjacent to the target cell. Exocytosis of CTL granule contents into the intercellular space between CTL and target cell allows perforin, a pore-forming protein found in the granules (2), to polymerize in the target cell membrane, thereby facilitating the entry of the granzymes, a family of serine proteases (3, 4), into the target cell, where they induce apoptosis.

Granzyme B (Cytotoxic Cell Proteinase-1/CCP1) represents a prototype of six of these serine proteases (granzymes B to G). Originally identified as a CTL-specific gene (5, 6) whose expression correlated with the induction of cytotoxicity (5), it was subsequently localized to the cytoplasmic granules of CTLs (7, 8). Quantitative polymerase chain reaction revealed that only granzyme B expression is correlated with the development of cytotoxicity in T cells in response to stimulation by mitogen, allogeneic cells, or CD3 (9), suggesting that it may be a direct effector in the lytic process. Shi et al. (10, 11) have shown that fragmentin-2, a serine protease purified from rat natural killer cell granules and having homology to granzyme B, is able to induce rapid DNA fragmentation, a hallmark of apoptosis, in YAC-1 target cells in the presence of perforin. Additionally, Heusel et al. (12) have shown that granzyme B is necessary for the early induction of DNA fragmentation and apoptosis in allogeneic target cells lysed by CTL (12), natural killer cells, or lymphokine-activated killer cells (13) from mice homozygous for a null mutation in the granzyme B gene.

Recent studies of apoptosis have shown that a family of cysteine proteases related to interleukin-1β-converting enzyme (ICE) play a key role. Originally identified as the protease responsible for producing biologically active IL-1β from the inactive precursor (14, 15), a role for ICE in the induction of apoptotic cell death was suggested by the discovery that casp-3, a gene required for programmed cell death in the nematode Caenorhabditis elegans, had homology to ICE (16). Indeed, overexpression of ICE or casp-3 in Rat-1 fibroblasts lead to apoptotic cell death of these cells (17). However, studies of mice deficient in ICE seemed to suggest that ICE does not play a nonredundant role in apoptosis but rather that other related proteases may be critical (18).

Multiple ICE-like proteases have now been isolated (reviewed in Ref. 19), including Ich1/Nedd-2 (20, 21), ICE/Ich-2/ICErelII (22–24), ICE/Ich-3 (24), Mch2 (25), Mch3/CBH-1/ICE-LAP3 (26–28), and CPP32/Apopain/Yama (29–31). These enzymes can be divided into three subfamilies: the ICE-like enzymes (ICE, TX/Ich-2/ICErelI, and ICErelIII), the CPP32-like proteases (CPP32, Ced-3, Mch2, and Mch3/CBH-1/ICE-LAP3), and the Nedd-2 proteins (Nedd-2 and Ich-1L). Each of these proteases is synthesized as an inactive precursor requiring cleavage after Asp residues for activation. When the ICE proteases were first discovered, it was postulated that granzyme B, which has substrate specificity requiring an Asp at P1
(32, 33), could induce target cell apoptosis by cleaving and activating an ICE protease (34). Although we found no evidence that granzyme B could cleave and activate ICE (35), we have recently shown that CPP32 is an intracellular substrate both in vitro and in vivo for granzyme B (36). Other workers have shown that granzyme B can cleave the CPP32-related protease CMH-1/Mch3/ICE-LAP3 in vitro (37), suggesting that granzyme B may act by activating this family of proteases. CPP32 (putative cysteine protease of 32 kDa) was identified as the protease responsible for cleavage of the nuclear protein poly-(ADP-ribose) polymerase during the induction of apoptosis (30, 31), as well as the catalytic subunit of DNA-dependent protein kinase and the U1 70-kDa small ribonucleoprotein (38).

Here we examine the ability of cytotoxic cells derived from mice deficient in granzyme B to induce CPP32 cleavage and apoptosis in target cells. Additionally, we use a peptide inhibitor of CPP32 to study its role in target cell DNA fragmentation. Our data demonstrate that CPP32 is cleaved and activated by granzyme B during CTL-mediated cytotoxicity in vivo. Furthermore, this pathway appears to be particularly relevant to DNA fragmentation rather than membrane damage.

MATERIALS AND METHODS

Cell Culture—Mice homozygous for a null mutation in the granzyme B gene have been previously described (12). Cytotoxic cells from these mice (GB KO) and from control mice (GB WT) were generated as follows. Splenocytes from these mice were activated in primary mixed lymphocyte cultures in the presence of IL-2 using irradiated splenocytes from Balb/c (H-2b) mice as stimulators for 3 days. Dead cells were removed by sedimentation through a Ficoll/metrizoate gradient. CTL21.9 is a cloned cytotoxic T cell line that requires antigenic stimulation (irradiated Balb/c splenocytes) and is dependent on IL-2 (39). All of the above cell lines were stimulated on a 7-day cycle with irradiated Balb/c splenocytes and were maintained in RPMI 640 supplemented with 5% (v/v) fetal calf serum, 100 μM β-mercaptoethanol (RHF) containing 60 units/ml recombinant IL-2. Effector cells were stimulated with anti-CD3 antibody (2C11, 1:500 dilution) for 24 h prior to cytotoxicity assays.

The L1210 (H-2b) variant transfected with mouse Fas cDNA (L1210-Fas) was kindly provided by Dr. Pierre Golstein, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Marseille, France (40). EL4 (H-2b) and YAC-1 (H-2b) mouse lymphomas and P815 (H-2a) mastocytoma cells were maintained by continuous culture in RHF. Chromium and [3H]Thymidine Release Assays—Cytotoxic activity, assessed by measuring 51Cr from labeled target cells, and DNA fragmentation, assessed by measuring [3H]thymidine release, were as described previously (39). Briefly, targets were labeled with 51Cr (DuPont NEN) for 1 h at 37°C or with [3H]thymidine (DuPont NEN) for 24 h at 37°C prior to the assays. Labeled targets were incubated with CTL21.9, GB KO, or GB WT effector cells at an effector to target ratio of 5:1 prior to the assays. Labeled targets were incubated with CTL21.9, GB KO, or GB WT effector cells at an effector to target ratio of 5:1 in a total volume of 100 μl in 96-well V-bottom plates for 2 h for preliminary studies or 4 h for inhibitor studies at 37°C. ConA was added to assays containing EL4, YAC-1, or L1210-Fas cells to a final concentration of 2 μg/ml. Ac-DEVD-CHO was used at a final concentration of 40 μM. CTL activity was calculated using the following formula: % lysis = 100 × (sample – spontaneous release)/(total – spontaneous release). Spontaneous release was determined by incubation of targets in the absence of CTL.

Western Blotting—Target cells (105) were incubated with appropriate numbers of effector cells at 37°C for 4 h, and then all cells were lysed in 10 mM Hepes/KOH, pH 7.4, 2 mM EDTA, 0.1% (v/v) CHAPS, 5 mM dithiothreitol. Cellular debris was removed by centrifugation, and then the entire lysate was resolved using 12% polyacrylamide SDS gels. Proteins were electroblotted to polyvinylidene difluoride and then probed using an antibody directed against the p17 subunit of CPP32. Detection was by probing with a horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence detection (Amersham Corp.).

RESULTS AND DISCUSSION

Recent work on apoptosis has focused on the role of the ICE/ced-3 cysteine proteases. We are specifically interested in the role of these proteases during CTL-mediated cytotoxicity. Initial studies of ICE proteases suggested that ICE itself could be a key mediator of apoptotic cell death (17), however, studies of mice lacking ICE revealed that ICE does not play a nonredundant role in apoptosis (18). Additionally, we have shown that ICE does not act as a substrate for granzyme B (35), a CTL-specific serine protease that is required for the early induction of target cell DNA fragmentation (10, 12). Subsequent studies revealed that granzyme B cleaves and activates the proteases CPP32 and CMH-1/Mch3/ICE-LAP3 during granule-mediated killing (36, 37).

To further study the role of CPP32 during granule-based CTL-mediated cytotoxicity, we first generated cytolytic cells from mice homozygous for a null mutation in the granzyme B gene (GB KO) or from control mice (GB WT). Previous studies using these mice have reported that DNA fragmentation is markedly decreased when effectors lacking granzyme B are used in comparison with control cells (12, 13). In order to confirm these results using our cell lines, we used 51Cr release (as a measure of membrane integrity) and [3H]thymidine release (as a measure of DNA fragmentation) from labeled YAC-1, EL4, P815, or L1210-Fas (a variant of L1210 transfected with the murine Fas cDNA) target cells as indicators of cytolytic activity. Fig. 1 confirms that the GB KO cells are clearly deficient at inducing DNA fragmentation in susceptible...
target cells when compared with control cells (Fig. 1B). In contrast, there is no apparent difference in induction of 51Cr release between these cell lines (Fig. 1A). The cytolytic activity (measured as 51Cr release) of these cells was reduced from 65 to 5% in the presence of EGTA, confirming that these cells kill predominantly through the granule pathway (data not shown).

We then assessed the ability of each of these effector cells to induce cleavage of CPP32 in target cells. Effector and target cells were combined at an effector to target ratio of 5:1 for 4 h at 37°C, and then all cells were lysed. Lysates were resolved by SDS-polyacrylamide gel electrophoresis and CPP32 was detected using an antibody directed against the large subunit of CPP32. The results of this study using EL4 and YAC-1 target cells are shown in Fig. 2 and indicate that CPP32 is not cleaved during CTL-mediated cytotoxicity in the absence of granzyme B (GB KO lanes). In contrast, CPP32 is completely cleaved to the active p17/p12 subunits in target cells exposed to control effectors (GB WT lanes, Fig. 2). Similar results were obtained using P815 cells as targets (data not shown). These results clearly show that granzyme B is required for the cleavage and activation of CPP32 during CTL-mediated cytotoxicity and confirm our previous finding that CPP32 may be an intracellular substrate for granzyme B (36).

If the incubation time of targets with effectors was increased to 24 h, CPP32 still was not cleaved in target cells in the absence of granzyme B (data not shown), further confirming that no other granzyme can substitute for this activity.

Together, our results suggest that granzyme B is involved in inducing target cell DNA fragmentation through the cleavage and activation of CPP32 (and probably CPP32-like proteases). To further test this hypothesis, we used the peptide inhibitor Ac-DEVD-CHO, which has previously been shown to inhibit CPP32 (30) and CMH-1/Mch3/ICE-LAP3 (26, 28) and may inhibit Mch2 (25), suggesting that it might inhibit all CPP32-like proteases. In contrast, Ac-DEVD-CHO cannot inhibit ICE (30).

In these studies, 51Cr and [3H]thymidine release from labeled L1210-Fas targets was measured in the presence of Ac-DEVD-CHO (40 μM) or dimethyl sulfoxide alone as a control, and then 51Cr (A) or [3H]thymidine (B) release was measured. Mean and standard deviation of triplicate samples are shown.

Having established that Ac-DEVD-CHO affects only [3H]thymidine release and not 51Cr release, we wondered whether ICE-like proteases also contribute to target cell DNA fragmentation and/or membrane damage. In these studies we used the prototypic ICE inhibitor Ac-YVAD-CHO. This inhibitor has been shown to inhibit ICE (15) and TX/Ich-2/ICErelII (23) but does not inhibit CPP32-like proteases (28, 30). Our results demonstrate that ICE-like proteases appear to play no role in the induction of target cell DNA fragmentation during granule-mediated killing. In a comparison between the effects of Ac-DEVD-CHO and Ac-YVAD-CHO on [3H]thymidine release, we found no inhibition by Ac-YVAD-CHO, even at the highest concentration used (100 μM, Fig. 4). However, Ac-DEVD-CHO had a striking effect on [3H]thymidine release, with maximal inhibition achieved by 50 μM but clear inhibition even at 5 μM (Fig. 4). These results suggest that ICE-like proteases play no role in the induction of target cell DNA fragmentation, whereas CPP32-like proteases are re-
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required for this process. We also found that ICE-like proteases play no role in the induction of target cell membrane damage. Ac-YVAD-CHO exerted no effect on $^{51}$Cr release from labeled targets when used in the assay at 40 μM (data not shown). These results are consistent with those of other workers who showed that inhibition of macrophage ICE activity by YVAD-chloromethylketone as demonstrated by suppression of mature IL-1β release had no effect on $^{51}$Cr release in response to CTL attack (41). Taken together, these inhibitor studies suggest the involvement of CPP32-like but not ICE-like proteases in the induction of DNA fragmentation during granule-mediated cytotoxicity. Furthermore, neither of these protease families appears to play a significant role in the induction of membrane damage following CTL attack.

In conclusion, we have confirmed that CPP32 is probably an intracellular substrate for granzyme B, and we have shown that this represents a nonredundant role for granzyme B. Additionally, the reduced ability of effector cells lacking granzyme B to induce rapid target cell DNA fragmentation can be accounted for by the failure of these cells to cleave and activate CPP32 or a CPP32-like protease because inhibition by Ac-DEVD-CHO also reduces target cell DNA fragmentation. However, neither the use of effector cells lacking granzyme B nor the presence of the CPP32 inhibitor had a significant effect on membrane damage, suggesting that both granzyme B and CPP32 are primarily involved in DNA fragmentation during CTL-induced apoptosis. The lack of effect of Ac-YVAD-CHO on either $^{31}$Cr or $^{3}H$thymidine release seems to suggest that ICE-like proteases are not required for granule-mediated cytotoxicity. It has recently been shown that during Fas-mediated killing, ICE-like proteases may be responsible for the activation of CPP32-like proteases (42). It is therefore likely that during granule-mediated killing, granzyme B replaces the function of ICE-like proteases by directly activating the CPP32 proteins. Because DNA fragmentation proceeds in the absence of granzyme B (Fig. 1) and in the absence of CPP32-like activity (Figs. 3 and 4), albeit with reduced efficiency, this suggests an alternate route to DNA fragmentation that does not involve CPP32-like proteins, possibly mediated by other granzymes.

Acknowledgments—We thank Irene Shostak and Rosemary Garner for technical assistance and Roger Bradley for preparation of photograph included in this manuscript.
Cleavage of CPP32 by Granzyme B Represents a Critical Role for Granzyme B in the Induction of Target Cell DNA Fragmentation

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J. Biol. Chem. 1996, 271:21709-21712.
doi: 10.1074/jbc.271.36.21709

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