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Caspase-dependent Ceramide Production in Fas- and HLA Class I-mediated Peripheral T Cell Apoptosis*

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We recently demonstrated that the engagement of HLA class I α1 domain induced Fas-independent apoptosis in human T and B lymphocytes. We analyzed the signaling pathway involved in HLA class I-mediated apoptosis in comparison with Fas (APO-1, CD95)-dependent apoptosis. The mouse mAb90 or the rat YTH862 monoclonal antibodies which bind the human HLA class I α1 domain induced the production of ceramide which was blocked by addition of the phosphatidylcholine-dependent phospholipase C inhibitor, D609. Furthermore, HLA class I-mediated apoptosis involved at least two different caspases, an interleukin-1 converting enzyme-like protease and another protease inhibited by the CPP32-like protease inhibitor Ac-DEVD-CHO. Despite similarity between Fas and HLA class I signaling pathways, we failed to demonstrate any physical association between these two molecules. We also report that the pan-caspase inhibitory peptide zVAD-fmk, but not Ac-DEVD-CHO and Ac-YVAD-CHO, inhibited decrease of mitochondrial transmembrane potential and generation of ceramide induced by anti-HLA class I and anti-Fas monoclonal antibodies, whereas all three peptides efficiently inhibited apoptosis. Altogether these results suggest that signaling through Fas and HLA class I involve caspase(s), targeted by zVAD-fmk, which act upstream of ceramide generation and mitochondrial events, whereas interleukin-1 converting enzyme-like and CPP32-like proteases act downstream of the mitochondria.

Apoptosis is a process that occurs in physiological or pathological conditions, in many different cell types, which serves as a fundamental control during the development of multicellular organisms. In the immune system, apoptosis induced by surface receptors is a central mechanism for the homeostasis of T and B lymphocytes.

With regard to human T cells, apoptosis can be triggered by several membrane receptors including three members of the tumor necrosis factor receptor (TNF-R)[1] family (1), Fas (CD95) (2–4), TNF-RI (5, 6), and CD30 (7), but also CD2 (8, 9), CD45 (10), HLA class I (11, 12), and CTLA4 (13) molecules. We recently described two monoclonal antibodies (mAbs) that bind to the α1 domain of HLA class I heavy chain and induce apoptosis of activated, but not resting, T and B lymphocytes (14, 15). Apoptosis induced by anti-HLA class I mAbs did not result from Fas/Fas-L interaction and distinct though partly overlapping populations of activated T cells were susceptible to Fas- and HLA class I-mediated apoptosis, respectively (15).

Regarding apoptosis signaling, TNF-RI and Fas have been shown to trigger rapid sphingomyelin hydrolysis into ceramide. Mutations in the TNF-RI cytoplasmic domain that abolished acidic sphingomyelinase and NF-κB activation in response to TNFα, also prevented cell death (16). Furthermore, in a Fas-resistant tumor cell line which expresses a death domain-defective Fas splice isoform, Fas ligation does not activate acidic sphingomyelinase although neutral sphingomyelinase and ERK-2 activities are still intact (17). In addition to TNFα and Fas-ligand (Fas-L), anti-IgM, ionizing radiation, heat shock, ultraviolet light, and oxidative stress (18–22) have been shown to induce ceramide production by cells in which they initiate apoptosis, suggesting that ceramide might be a general mediator of apoptosis. Recent studies have provided compelling evidence that a cascade of Asp-directed cysteine proteases, renamed caspases (23), plays a pivotal role in transduction of apoptotic signals. Particularly, interleukin-1 converting enzyme (ICE)-like proteases have been implicated in Fas- and TNFα-induced cell death (24–29). Caspases can be clustered into three groups according to their specificities and their biological functions: group I/ICE (caspase-1), TX/ICH2/ICE_{rel}-II (caspase-4), ICE_{rel}-III/TY (caspase-5), group II/Yama/CPP-32/apopain (caspase-3), Mch3/ICE-LAP3/CMH-1 (caspase-7), and 3/ICH-1/Nedd2 (caspase-2), and group III/Mch2 (caspase-6), MACH/FLICE/Mch5 (caspase-8) ICE-LAP-6/Mch6 (caspase-9), and Mch4 (caspase-10) (reviewed in Refs. 23 and 30). The specificity of the caspase family has been defined by cleavage sequences in their respective substrates, which permitted the generation of specific inhibitory peptides, such as Ac-DEVD-CHO, which inhibits CPP32-like protease activity but also other members of caspase family (31), Ac-YVAD-CHO, a specific inhibitor of ICE-like protease activity (27), and Ac-zVAD-fmk, a pan-caspase inhibitor.

The relationship between ceramide production and caspases

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[1] The abbreviations used are: TNF-R, tumor necrosis factor receptor; CsA, cyclosporin A; $\Delta\psi_m$, mitochondrial transmembrane potential; Fas-L, Fas ligand; ICE, interleukin-1 converting enzyme; mAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PC-PLC, phosphatidylcholine-specific phospholipase C; PARP, poly(ADP-ribose) polymerase; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate.

activation in apoptosis signaling is still not well defined. Recently, a control of ceramide production by caspases has been proposed in REAPER- and TNFα-induced apoptosis models (32, 33), but remains to be demonstrated in other models of apoptosis.

In the present study we investigated the HLA class I signaling pathway leading to apoptosis of activated T lymphocytes, and we examined the possible connection between ceramide and caspases in both Fas- and HLA class I-apoptosis pathways. Our results point toward activation of caspases upstream and downstream of ceramide production, leading to reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) and subsequent propagation of the death signal.

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture—Peripheral blood lymphocytes (PBL) were collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, then mononuclear cells were isolated by centrifugation on a layer of Histopaque® (Sigma). Those cell suspensions referred to as PBL contained 1.8 ± 0.4% monocytes as defined by expression of CD14. PBL were resuspended in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). Cells (1×10^6/ml) were incubated in the presence of phytohemagglutinin (PHA), 5 μg/ml (Sigma). Cultures were maintained in a humid atmosphere containing 5% CO_2 for 72 h.

Antibodies and Reagents—MAb90 (anti-HLA-A, -B, -C, mouse IgG_1) was produced as described previously (15) and purified from ascites fluids by DEAE chromatography. The YTH862 mAb (anti-HLA-A, -B, and -C, rat IgG2b) was produced in Prof. H. Waldmann's laboratory (Oxford, United Kingdom). W6/32 mAb (anti-HLA-A, -B, and -C, IgG_{2a}) was obtained from ATCC (Rockville, MD). Epitope specificity of the different anti-HLA class I antibodies was analyzed by using transfected C1R cells that express HLA-A2.1 or hybrid mouse/human exon-shuffled HLA-A2.1/H-$2D^d$ genes (34) and has been previously reported (15). Briefly, mAb90 and YTH862 bind to closely related or identical epitopes of the α1 domain, whereas W6/32 binds to a non-polymorphic epitope of the α2 and α3 domains. Purified anti-Fas mAb agonist (IgM, clone CH11) and FITC anti-Fas mAb (IgG_1, clone UB2) were purchased from Immunotech (Marseille, France). IgG_1 control mAb (for bioassays) and FITC IgG control mAbs (for flow cytometric analyses) were from Dako (Glostrup, Denmark). The phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D609, genistein, chelerythrine, okadaic acid, and cytochalasin B and D were purchased from Sigma. The tetrapeptide ICE-like protease inhibitor Ac-YVAD-CHO and CPP32-like protease inhibitor Ac-DEVD-CHO were from Neosystem (Strasbourg, France), zVAD-fmk was from Bachem (Voisins le Bretonneux, France). Cyclosporin A (CsA) was kindly supplied by Sandoz Corp. (Basel, Switzerland).

Measurement of Apoptosis—After 3 days of culture, PHA-activated PBL were harvested. Dead cells were removed by centrifugation on a layer of Histopaque (Sigma) and washed in Hank's balanced salt solution. Viable cells (10^6/ml) were incubated in 96-well microplates with various inhibitors and then treated with mAb90. After 24 h of incubation, cell death was evaluated by fluorescence microscopy after staining with Hoechst 33342 (Sigma) at 10 μg/ml following previously described methods (35). Nuclear fragmentation and/or marked condensation of the chromatin with reduction of nuclear size were considered as typical features of apoptotic cells. Based on these measurements, results were expressed as percentage of specific apoptosis according to the following formula, % specific apoptosis = (% of apoptotic treated cells − % of apoptotic control cells) × 100/100 − of apoptotic control cells.

Evaluation of Mitochondrial Transmembrane Potential—To evaluate the mitochondrial transmembrane potential (36), cells (2×10^5/ml) were incubated with 3,3′-dihexyloxacarbocyanine (3), 40 nM in phosphate-buffered saline (Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C, followed by analysis on a cytofluorometer (Becton Dickinson, Mountain View, CA; λ excitation maximum 488 nM, λ emission maximum 525 nm).

Ceramide Assay—Ceramide was quantified by the diacylglycerol kinase assay as ^{32}P incorporated upon phosphorylation of ceramide to ceramide 1-phosphate by diacylglycerol kinase from *Escherichia coli* (Biomol, Plymouth Meeting, PA) (37). Briefly, after 3 days of culture, 2×10^7 PBL were starved for 2 h in RPMI containing 2% bovine serum albumin and then treated with the different mAbs for indicated times. Ceramide 1-phosphate was resolved by TLC using $CHCl_3/CH_3OH/CH_3COOH$ (65:15:5, v/v) as solvent. Authentic ceramide 1-phosphate was identified by autoradiography at R_F 0.25. The level of ceramide was determined by comparison to concomitantly run standard curve comprised of known amounts of ceramide (Sigma) and normalized to [^{3}H]triglyceride introduced during lipid extraction.

ICE-like Protease Activity Assay—Three-day PHA-stimulated PBL were permeabilized using the osmotic shock method as followed: 10^7 PBL/ml were incubated with 50 μM ICE substrate DABCYL-YVADAPV-EDANS (Bachem, Voisins le Bretonneux, France) and distilled water (1:1, v/v). After 5 min incubation at 37 °C, appropriate volumes of 5 × phosphate-buffered saline in 50% fetal calf serum were added to bring the osmolarity to normal level. Then cells (10^6/ml) were incubated with different mAbs for indicated times and analyzed by FACS Star Plus analysis (Becton Dickinson, Pont de Claix, France) using an excitation wavelength of 360 nm and emission wavelength of 488 nm.

Determination of Poly(ADP-ribose) Polymerase (PARP) Cleavage—Cleavage of PARP was determined by Western blotting. The cells (1×10^6) were washed twice in phosphate-buffered saline, pelleted, and lysed in 100 μl of lysis buffer containing 62.5 mM TRIS, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol. After 10 min on ice, 20 μl of 5 × sample buffer was added, and samples were heated for 5 min at 95 °C. Thirty μl of the lysate were subjected to 7.5% SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane. Blots were probed using anti-PARP mAb C-2-10 (Biomol, TEBU, Le Perray en Yvelines, France). Bound antibodies were detected with rabbit anti-mouse peroxidase-conjugated antibodies (Bio-Rad, Ivry sur Seine, France). An enhanced chemiluminescence system (Amersham, France) was used for detection.

Fluorescence Energy Transfer Experiments—Three-day PHA-activated PBL were stained with tetramethylrhodamine isothiocyanate-conjugated (TRITC) mAb90 and FITC anti-Fas mAb UB2 or FITC anti-CD25 mAb. Fluorescence energy transfer experiments have been done by measurement of decrease fluorescence anisotropy by a confocal microscope Acas 570 (Meridian) as described previously (38, 39). When resonance energy transfer occurred between a donor-acceptor pair, the following phenomena could be observed: quenching of the fluorescence emission of the donor molecule; sensitization of the emission of the acceptor; and a reduction in the anisotropy of the acceptor emission, which was dependent on the ratio of excitation through direct energy transfer from the donor to the acceptor.

RESULTS

Inhibition of HLA Class I-mediated Apoptosis by Cytochalasins and Okadaic Acid—A series of inhibitors of various activation pathways was used for comparison of Fas and HLA class I-mediated apoptosis. PBL from healthy donors were activated for 3 days with PHA and then treated with various inhibitors before exposure to mAb90, YTH862, or the anti-Fas mAb CH11. Genistein, chelerythrine, EGTA, or CsA failed to interfere with HLA class I and Fas-mediated apoptosis, suggesting that neither protein tyrosine kinase nor PKC activation, Ca^{2+} influx, or phosphatase 2B were involved in HLA class I apoptotic signaling (Fig. 1*A*). In contrast, okadaic acid which inhibits phosphatase 1, 2A, and 2C significantly decreased HLA class I- and Fas-mediated apoptosis (Fig. 1, *A* and *B*). The inhibition of mAb 90 and YTH862-induced apoptosis was dose-dependent and reached 90% at 10 ng/ml (Fig. 1*B*). Unlike Fas-, HLA class I-mediated cell death was strongly inhibited by cytochalasin B and D (Fig. 1, *A* and *C*), suggesting that HLA class I but not Fas-dependent apoptosis requires cytoskeletal rearrangement and possibly association with other molecules.

MAb90 and YTH862 Induce Ceramide Production under the Control of a Phosphatidylcholine-specific Phospholipase C—Okadaic acid has been reported to inhibit ceramide-induced apoptosis, possibly by blocking a ceramide-activated protein phosphatase which belongs to the heterodimeric subfamily of the phosphatases 2A group (40). Since okadaic acid inhibits HLA class I-induced apoptosis (Fig. 1, *A* and *B*), we investigated whether HLA class I engagement would generate ceramide production. Lipids were extracted from PHA-activated PBL treated by W6/32, mAb90, YTH862, anti-Fas mAb CH11 or control IgG_1, and endogenous ceramide production was measured by a diacylglycerol kinase assay. While W6/32 and control IgG_1 did not induce ceramide production, the two anti-HLA

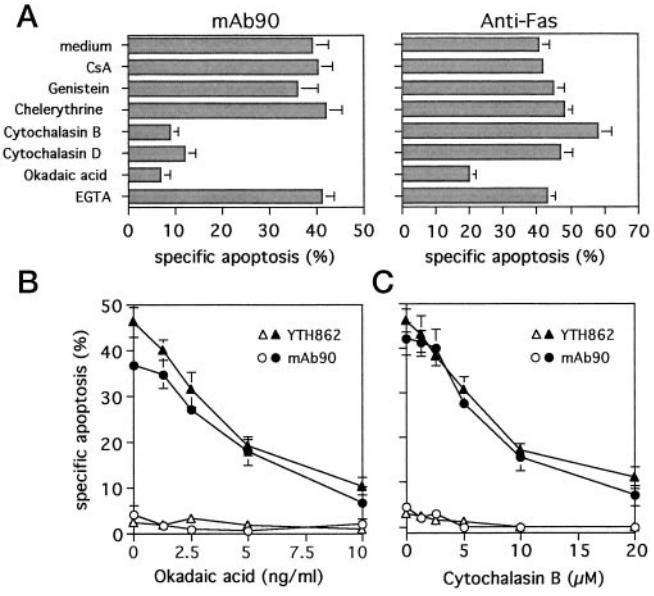

FIG. 1. **Effect of various inhibitors on HLA class I- and Fas-induced apoptosis.** *A*, 3-day PHA-activated PBL were preincubated with CsA (250 ng/ml) for 3 h, or genistein (100 μM), chelerythrine (1 μM), cytochalasin B (20 μM), cytochalasin D (10 μg/ml), okadaic acid (10 ng/ml), and EGTA (500 μM) for 1 h before addition of mAb90 (10 μg/ml) or the anti-Fas mAb CH11 (1 μg/ml). *B* and *C*, resting PBL (*open symbols*) or 3-day PHA-activated PBL (*closed symbols*) were preincubated with okadaic acid or cytochalasin B for 1 h. After preincubation, mAb90 (10 μg/ml) or YTH862 (1/20) were added for 24 h and the percentage of apoptotic cells was determined by fluorescent microscopy after staining with Hoechst 33342. Results are expressed as mean ± S.E. of four independent experiments.

class I α1 domain, mAb90 or YTH862 which trigger apoptosis, increased the basal level of ceramide production by 159 and 166%, respectively (Fig. 2*A*). As control, anti-Fas mAb increased the basal level of ceramide production by 162%. Ceramide production by mAb 90 was obtained after 10 min and decreased rapidly thereafter (Fig. 2*B*). We also tested an inhibitor of PC-PLC, the xanthate D609 which subsequently blocks the activation of acidic sphingomyelinase but not neutral sphingomyelinase (17, 18) on HLA class I-induced ceramide production. Results in Fig. 2*C* show that D609 reduced the production of ceramide induced by mAb90 to the control level, indicating that HLA class I-mediated ceramide generation may depend upon PC-PLC activation.

Anti-HLA Class I mAbs Activate ICE-like Proteases and Induce PARP Cleavage—Several studies have demonstrated the activation of caspases in different pathways of apoptosis (25, 26, 31). To directly address the involvement of ICE-like proteases in HLA class I-mediated apoptosis, we studied the kinetics of ICE-like protease activity after anti-HLA class I treatment. Permeabilized PHA-activated PBL were incubated with the fluorogenic ICE substrate DABCYL-YVADAPV-EDANS, which contains the cleavage site of interleukin-1β precursor (41) and then treated with W6/32, mAb90, YTH862, and anti-Fas mAb CH11. As shown in Fig. 3*A*, mAb90, YTH862, and anti-Fas mAb induced ICE-like protease activity with a peak at 1 h for anti-HLA class I mAbs and 2 h for anti-Fas mAb. In parallel, cleavage of PARP to its signature 89-kDa fragment (42) was studied by immunoblotting. PHA-activated PBL were treated for 0, 2, and 6 h in the presence of CH11, mAb90, or IgG_1 as a control, PARP cleavage was evident within 2 h with both mAb90 and the anti-Fas mAb CH11, but not with control IgG_1 (Fig. 3*B*). These results suggest that, like Fas cross-linking, HLA class I-mediated apoptosis involves activation of at least ICE-like and CPP32-like family proteases.

Inhibition of Caspases Prevents HLA Class I-mediated Apoptosis—Caspases are specifically inhibited *in vitro* and *in vivo* by cell-permeable tetrapeptides designed to mimic cleavage sites of their respective substrates. We used such inhibitory peptides to test whether caspases, other than ICE-like proteases, are involved in HLA class I-mediated apoptosis. Preincubation of PHA-activated PBL, for 3 h, with zVAD-fmk, Ac-DEVD-CHO, or Ac-YVAD-CHO, before treatment with antibodies, inhibited both HLA class I- and Fas-mediated apoptosis (Fig. 4). However, Ac-DEVD-CHO significantly decreased apoptosis at 1 μM and achieved nearly complete (96%) inhibition of HLA class I, but partial (75%) diminution of Fas-mediated apoptosis at 100 μM (Fig. 4*B*), while Ac-YVAD-CHO was not or weakly active at 1 μM and inhibited about 50 to 60% of apoptosis at 100 μM (Fig. 4*A*). zVAD-fmk was the most efficient inhibitor, reducing by 50–80% at 1 μM and 100% at 100 μM both HLA class I- and Fas-mediated apoptosis. These results suggest that HLA class I-mediated apoptosis involves at least two different caspases, an ICE-like protease and another protease(s) inhibited by the CPP32-like inhibitor Ac-DEVD-CHO.

HLA Class I Molecules Do Not Associate with Fas—Since HLA class I molecules may associate with other cell surface functional molecules such as the interleukin-2 receptor (43), it could be hypothesized that HLA class I may trigger apoptosis by associating itself with death signaling molecules such as Fas. This type of association might be induced by PBL activation or, alternatively, only by engagement of HLA class I molecules with mAbs which induce apoptosis, such as mAb 90 and YTH862. To investigate these hypotheses, fluorescence energy transfer experiments have been performed by measurement of fluorescence anisotropy after incubation of PHA-activated PBL with TRITC-conjugated mAb90 or W6/32 and FITC-conjugated anti-Fas mAb UB2 or anti-CD25 mAb as positive control. When two molecules are physically associated, resonance energy transfer occurs between the donor-acceptor pair, which leads to the quenching of fluorescence emission by the donor molecule, sensitization of the emission by the acceptor, and a reduction in the anisotropy of the acceptor molecule. Results in Table I show that FITC anti-CD25 mAbs decrease the fluorescence anisotropy induced by TRITC-mAb90 or TRITC-W6/32 alone confirming the association between HLA class I and IL-2 receptor, whereas FITC anti-Fas mAbs do not. Moreover, mAb90 precipitated the 45-kDa heavy chain and 12-kDa β2m from the surface biotinylated PHA blasts but not a 48-kDa protein corresponding to Fas (data not shown). These results show that HLA class I molecules do not associate with Fas upon PHA activation or upon binding of the apoptosis-inducing mAb90, further excluding a contribution of cell surface Fas molecules in HLA class I-mediated apoptosis, and reinforcing the hypothesis of an intracellular interaction between Fas and HLA class I signaling cascades.

zVAD-fmk, but Not Ac-DEVD-CHO or AC-YVAD-CHO, Inhibits Ceramide Production Induced by Anti-Fas and Anti-HLA Class I mAbs—Knowing that the peptides zVAD-fmk, Ac-DEVD-DHO, and Ac-YVAD-CHO inhibit both HLA class I- and Fas-mediated apoptosis, we tested whether these three inhibitory peptides would also inhibit ceramide production. In these experiments PHA-activated PBL were treated for 2 h with each peptide at 100 μM before the addition of mAb90, YTH862, or CH11 mAbs. After 10 min in the presence of CH11, mAb90, or YTH862 mAbs, phospholipids were extracted and endogenous ceramide production was measured by a diacylglycerol kinase assay. As shown in Fig. 5, zVAD-fmk, but not Ac-YVAD-CHO or Ac-DEVD-CHO, inhibited ceramide production induced by either anti-Fas or anti-HLA class I mAbs. Pretreatment of cells

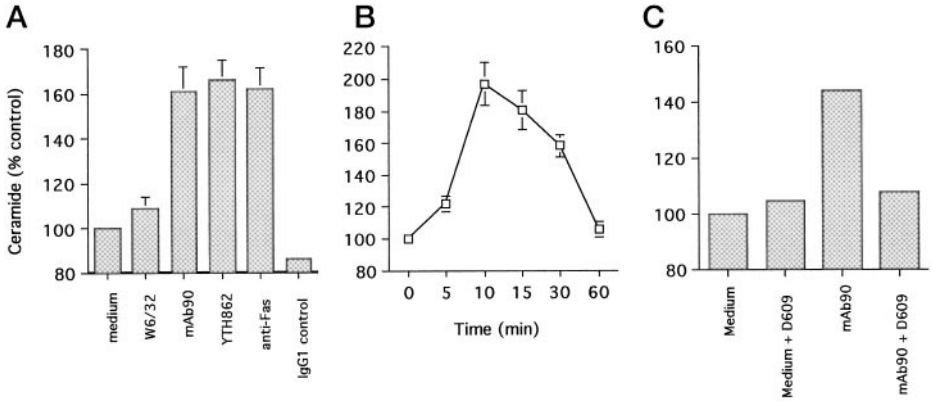

FIG. 2. *A*, effect of different anti-HLA class I mAb on ceramide production. Three-day PHA-activated PBL were incubated in the presence of medium alone, W6/32 (10 μg/ml), mAb 90 (10 μg/ml), YTH862 (1/20), control IgG_1 (10 μg/ml), or the anti-Fas mAb CH11 (1 μg/ml) for 10 min. *B,* time course of ceramide production by mAb90. Three-day PHA-activated PBL were incubated in the presence of mAb90 (10 μg/ml) for the indicated times. *C*, effect of D609 on HLA class I-induced ceramide production. Three-day PHA-activated PBL were incubated for 10 min with mAb90 at 10 μg/ml, after 30 min preincubation with 25 μg/ml D609. *A-C*, quantitation of ceramide production was performed by diacylglycerol kinase assay after lipid extraction. Results are expressed as mean ± S.E. of three independent experiments for *panels A* and *B* and data from a representative experiment among two showing similar results are shown in *panel C*.

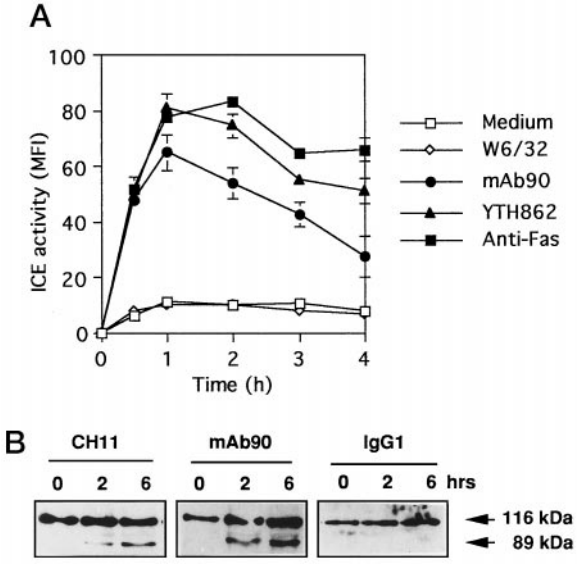

FIG. 3. **Stimulation of ICE-like and CPP32-like proteolytic activity by mAb90 and YTH862.** *A*, 3-day PHA-activated PBL were incubated with the fluorogenic ICE substrate DABCYL-YVADDAP-EDAMS (50 μM) and then treated for indicated times with medium alone, W6/32 (10 μg/ml), mAb90 (10 μg/ml), YTH862 (1/20), or the anti-Fas mAb CH11 (1 μg/ml). Results were obtained by fluorescence-activated cell sorter analysis and expressed as mean fluorescence intensity units after subtraction of spontaneous fluorescence. Results are expressed as mean ± S.E. of three independent experiments. *B*, 3-day PHA-activated PBL were treated for the indicated times with mAb90 (10 μg/ml), the anti-Fas mAb CH11 (1 μg/ml), or control IgG_1 (10 μg/ml). After lysis proteins were separated by SDS-polyacrylamide gel electrophoresis followed by Western blotting with the anti-PARP mAb C-2-10 as described under "Experimental Procedures."

with zVAD-fmk (100 μM) resulted in 60–70% inhibition of ceramide production induced by CH11 and mAb90 mAbs, and 34% inhibition in the case of YTH862 mAb (Fig. 5*A*). A dose-response experiment with the zVAD-fmk inhibitory peptide on ceramide production induced by CH11 mAb showed a very weak inhibition at 1 μM (<10%), whereas at 10 or 100 μM zVAD-fmk reduced ceramide production to the level observed in presence of the tetrapetide alone (Fig. 5*B*). In contrast pretreatment with either Ac-YVAD-CHO or AC-DEVD-CHO did not block Fas- and HLA-class I-induced ceramide production, but significantly increased it (Fig. 5, *C* and *D*). These results show that the generation of ceramide requires at least one functional caspase which could be different from ICE-like or CPP32-like proteases.

zVAD-fmk, but Not Ac-DEVD-CHO or Ac-YVAD-CHO, Inhibits Reduction of Mitochondrial Transmembrane Potential Induced by Anti-Fas and Anti-HLA Class I mAbs—Before cells exhibit signs of nuclear apoptosis (chromatin condensation and DNA fragmentation), they undergo a reduction of $\Delta\Psi_m$, due to the opening of mitochondrial permeability transition pores (44). We tested the effect of the three inhibitory peptides on $\Delta\Psi_m$ modification induced in both HLA class I and Fas apoptosis. Pretreatment of PHA-activated PBL, for 3 h, with 100 μM zVAD-fmk, before addition of CH11 or mAb90 significantly reduced $\Delta\Psi_m$ disruption induced by both CH11 and mAb90 (Fig. 6). The protective effect of zVAD-fmk on $\Delta\Psi_m$ was not observed with similar doses of Ac-DEVD-CHO or Ac-YVAD-CHO. However, the three tetrapeptides efficiently inhibited chromatin condensation and nuclear fragmentation evaluated by fluorescence microscopy after staining with Hoechst 33342 (Fig. 4). Finally $\Delta\Psi_m$ disruption induced by addition of exogenous C2-ceramide, but not the inactive control DHC2, was not inhibited by any of the peptides (Fig. 6). These results suggest that zVAD-fmk targets a protease which acts upstream of ceramide generation and mitochondrial events, whereas ICE-like and CPP32-like proteases act downstream of mitochondria (Fig. 7).

DISCUSSION

This study was undertaken to define the molecular events involved in HLA class I-mediated apoptosis and examine the relationship between caspases and ceramides, two mediators implicated in different apoptosis pathways. We show here that cell death induced by HLA class I molecules involves activation of caspases and ceramide production, similarly to what is observed during Fas apoptosis, despite the fact that HLA class I-mediated apoptosis is independent of Fas/Fas-L interaction (15). Recently two other reports described induction of apoptosis by HLA class I molecules (11, 12). Apoptosis induced by the anti-HLA class I α3 domain, 5H7, was not inhibited by zVAD-fmk and was triggered in cell lines derived from patients with Niemann-Pick disease that lack acidic sphingomyelinase activity, suggesting that neither ceramide nor caspases were involved in 5H7-mediated apoptosis (11). Furthermore, Skov *et al*. (12) demonstrated that herbimycin A inhibits rabbit anti-β2m-induced apoptosis of Jurkat T cells, suggesting that TPK might be involved in this type of apoptosis. The difference with our results may be explained by the fact that our antibodies

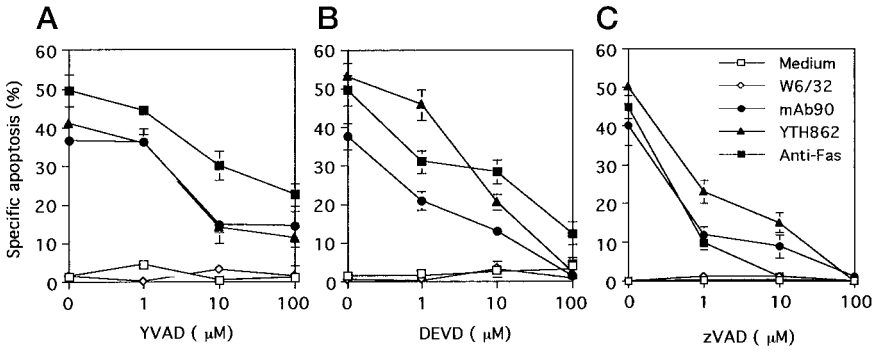

FIG. 4. **Involvement of ICE-like and CPP32-like proteases in HLA class I-mediated apoptosis.** Three-day PHA-activated PBL were preincubated for 3 h in the presence of the peptide Ac-YVAD-CHO (*A*), Ac-DEVD-CHO (*B*), or zVAD-fmk (*C*), at the indicated concentrations before addition of medium alone, W6/32 (10 μg/ml), mAb90 (10 μg/ml), YTH862 (1/20), or the anti-Fas mAb CH11 (1 μg/ml). After 24 h the percentage of apoptotic cells was determined by fluorescent microscopy after staining with Hoechst 33342. Results are expressed as mean ± S.E. of four independent experiments.

TABLE I
HLA class I molecules do not associate with Fas

Three-day PHA-activated PBL were incubated for 30 min with TRITC-mAb90 or TRITC-W6/32 at 10 μg/ml in the presence or absence of FITC anti-CD25 or anti-Fas mAbs. Fluorescence energy transfer was measured by decrease of fluorescence anisotropy as described under "Experimental Procedures."

	Medium	Anti-CD25 FITC	Anti-Fas FITC
mAb90 TRITC	0.397 ± 0.008[a]	0.079 ± 0.015	0.367 ± 0.011
W6/32 TRITC	0.412 ± 0.012	0.092 ± 0.013	0.409 ± 0.007

[a] Fluorescence anisotropy.

(mAb90 and YTH862) recognize the α1 domain of HLA class I molecules and do not need to be cross-linked to induce apoptotic cell death (15).

Several reports have demonstrated that activation induced by cross-linking of HLA class I molecules depends on an association between these molecules and other cell surface proteins such as interleukin-2R or CD3/TCR (45). We further observed that inhibition of cytoskeleton rearrangement by cytochalasin B or D, which may inhibit association of cell surface molecules, prevents HLA class I- but not Fas-mediated apoptosis. However, HLA class I-induced apoptosis seems to be independent of associated cell surface molecules. Indeed we showed that monovalent Fab′ fragments from mAb90 were nearly as efficient as intact IgG to induce apoptosis (15). Furthermore, using two different methods, energy transfer and immunoprecipitation, we failed to demonstrate any physical association between HLA class I and Fas molecules. This argues in favor of a death signal transduced by the HLA class I molecule itself which differ from Fas-mediated apoptosis at least by cytoskeleton rearrangement requirement. The cytoplasmic region of the α chain of the HLA class I molecule which comprises 33 amino acids does not bear the typical death domain found in the intracellular C terminus region of both Fas and TNF-RI. So we can speculate that HLA class I molecules will interact with specific intracellular proteins that can initiate cell killing, and then converge in a route common with Fas. HLA class I-specific associated proteins that are involved in proximal events which control HLA class I cell death signaling remain to be identified.

The two anti-HLA class I mAbs which induce apoptosis, mAb90 and YTH862, increased ceramide levels as did the anti-Fas mAb (Fig. 2, Ref. 17). Evidence for the requirement of a PC-PLC/acidic sphingomyelinase pathway in this process was suggested by using the xanthate D609 which inhibits ceramide production induced by Fas (17) and anti-HLA class I mAbs. Indeed D609 specifically blocks the PC-PLC and subsequent activation of acidic sphingomyelinase but not neutral sphingomyelinase (46, 47). The complete inhibition of ceramide production in the presence of D609 suggests that anti-HLA class I mAbs activate only acidic sphingomyelinase and not neutral sphingomyelinase. The major role for ceramide in apoptosis signaling was recently demonstrated by genetic models. Santana *et al.* (48) reported that lymphoblasts from Niemann-Pick patients, which bear an inherited deficiency of acidic sphingomyelinase activity, as well as cells from acidic sphingomyelinase knockout mice, failed to respond to ionizing radiations with ceramide generation and apoptosis. Finally our results

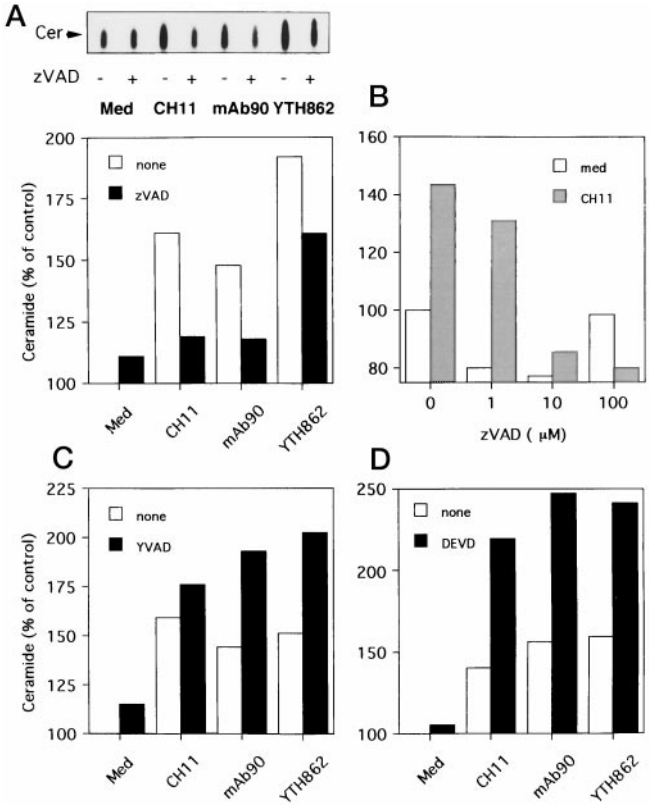

FIG. 5. **Effect of zVAD-fmk, Ac-DEVD-CHO, and Ac-YVAD-CHO on ceramide production induced by anti-Fas and anti-HLA class I mAbs.** Three-day PHA-activated PBL were preincubated 2 h in the presence of the peptide zVAD-fmk (*A*), Ac-YVAD-CHO (*C*), or Ac-DEVD-CHO (*D*) at 100 μM before addition of medium alone, mAb90 (10 μg/ml), YTH862 (1/20), or the anti-Fas mAb CH11 (1 μg/ml) for 10 min. In *B*, 3-day PHA-activated PBL were preincubated 2 h in the presence of the peptide zVAD-fmk at the indicated concentrations before addition the anti-Fas mAb CH11. Quantitation of ceramide production was performed by diacylglycerol kinase assay after lipid extraction. Data from a representative experiment among two showing similar results are shown.

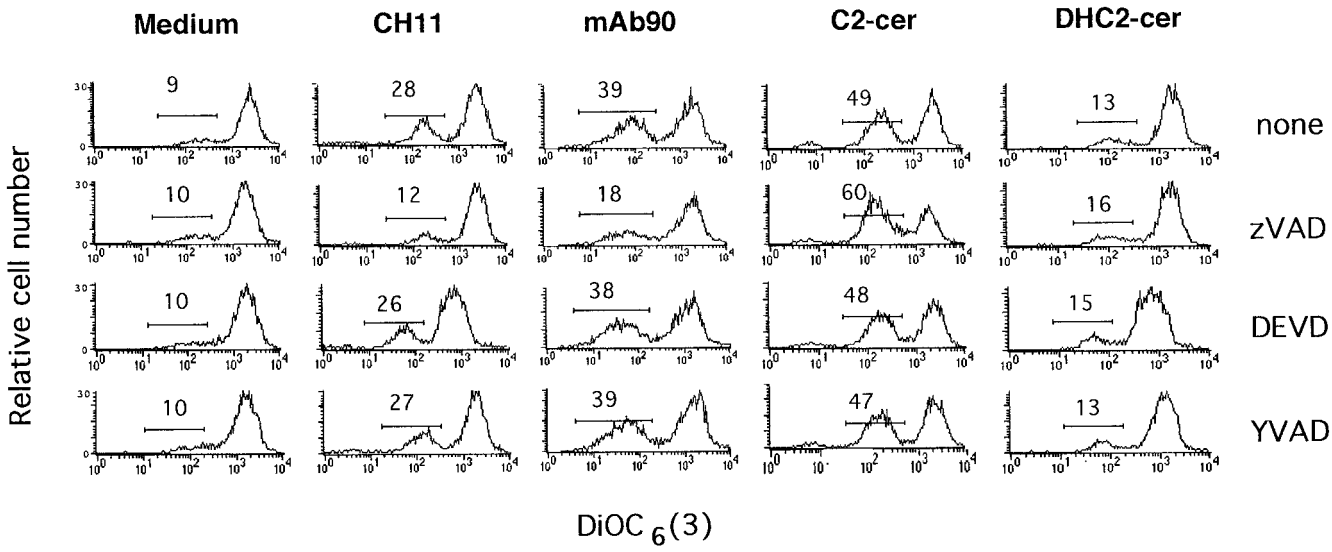

FIG. 6. **Effect of zVAD-fmk, Ac-DEVD-CHO, and Ac-YVAD-CHO on decrease mitochondrial membrane potential (ΔΨm).** Three-day PHA-activated PBL were preincubated 3 h in the presence of the peptides zVAD-fmk, Ac-YVAD-CHO, or Ac-DEVD-CHO at 100 μM before addition of medium alone, anti-Fas mAb CH11 (1 μg/ml), mAb90 (10 μg/ml), C2-ceramide, or DHC2-ceramide (100 μM). After 12 h, $\Delta\Psi_m$ modifications were evaluated by staining with 3,3′-dihexyloxacarbocyanine (*$DiOC_6(3)$*). The percentage of cells with decreased mitochondrial membrane potential are indicated for each histogram.

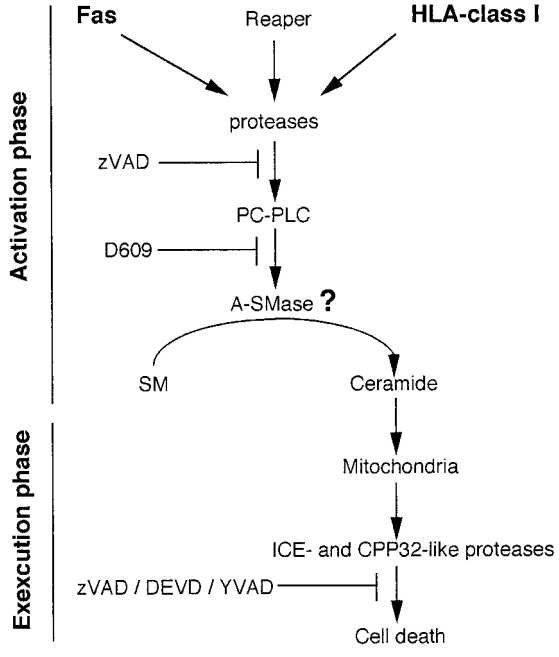

FIG. 7. **Schematic presentation of the proposed connection between caspases and ceramide in Fas and HLA class I signaling pathway.** Sites of inhibition of the three inhibitory peptides (zVAD-fmk, Ac-YVAD-CHO, and Ac-DEVD-CHO) and D609 are indicated.

extend the list of apoptotic stimuli which use ceramides for the propagation of their death signal since TNFα (18), Fas-L (17, 19), anti-IgM (20), and stress-induced apoptosis (21, 22) increase ceramide levels.

It is clear that caspases play a major role in apoptosis signaling. Expression of *crmA*, a cowpox virus encoding a serpin that is a specific inhibitor of ICE-like proteases, suppressed TNFα-, Fas-, and granzyme B-induced apoptosis (28, 29, 49). By measuring the cleavage of a fluorogenic substrate specific for ICE-like proteases as well as PARP cleavage, and using synthetic inhibitors specific for ICE-like (Ac-YVAD-CHO) and CPP32-like (Ac-DEVD-CHO) proteases, we found that these two caspases subfamilies that are involved in Fas-mediated apoptosis, are also activated during HLA class I-induced apoptosis. More importantly our data demonstrate that the large spectrum caspases inhibitor, zVAD-fmk, inhibits ceramide production induced by both anti-HLA class I and anti-Fas mAbs, suggesting that zVAD-fmk targets (a) protease(s) acting upstream of ceramide generation. In agreement with this hypothesis zVAD-fmk has no effect on the decrease of mitochondrial transmembrane potential induced by addition of exogenous ceramide (Fig. 6). Control of ceramide production by caspases was already proposed in other models. The *Drosophila melanogaster* protein REAPER, which is critical for the *Drosophila* embryo, was found to induce generation of ceramide and apoptosis, both being blocked by the zVAD-fmk peptide (32). More recently it was demonstrated that Ac-YVAD-CHO and CrmA, two potent inhibitors of ICE-like proteases, inhibited ceramide generation and prevented TNF-α-induced cell death in MCF-7 breast carcinoma cells (33). Despite the observation that, in Fas- and HLA class I-mediated apoptosis, a different pattern of inhibition is obtained with the tetrapeptide Ac-YVAD-CHO compared with TNF-α-mediated apoptosis, our results support the role of caspases in controlling ceramide generation in cell death signaling. Furthermore, the strong accumulation of ceramides observed when cells were pretreated with either Ac-DEVD-CHO or Ac-YVAD-CHO suggests that blockade of ICE-like or CPP32-like proteases inhibits or delays ceramide consumption. It was recently reported that CD95- and ceramide-induced apoptosis requires G_{D3} ganglioside, newly synthesized from ceramide (50). The authors documented that a DEVD- and/or YVAD-sensitive caspase controls GD3 accumulation. According to these data, a possible control of GD3 synthase by caspases can be proposed and certainly deserves further investigations.

A possible candidate for the proximal caspase targeted by zVAD-fmk peptide, could be the recently identified FLICE protease (51, 52) proposed to be the most receptor-proximal caspase activated in Fas signaling (53, 54). The recruitment of FLICE in the death-inducing complex is mediated by the N terminus prodomain of the molecule, which interacts with the death effector domain of FADD. However, FLICE was also shown to be activated by granzyme B (51), indicating that

FLICE can be recruited and activated by proteins which do not possess a death domain. Whether FLICE is also activated in HLA class I-mediated apoptosis remains to be demonstrated. Recent data from Medema *et al.*, (53) on the B lymphobastoid cell line SKW6.4 showed that in an *in vitro* cleavage assay, where ^{35}S-labeled FLICE is added to immunoprecipitates of the death-inducing signaling complex, autocatalytic cleavage of FLICE is blocked by the peptide inhibitors zVAD-fmk or DEVD-fmk but not by CrmA or Ac-YVAD-CHO. In the cell, however, inhibition of FLICE was only documented with zVAD-fmk. It is possible that DEVD-fmk (or Ac-DEVD-CHO) would not inhibit FLICE activity *in vivo* because of a higher affinity for other proteases such as CPP32. This would explain why we did not see any effect of the tetrapeptide Ac-DEVD-CHO on ceramide generation, despite its capacity to inhibit HLA class I and Fas-mediated apoptosis.

According to our results, we propose a model (Fig. 7) where a first set of proteases targeted by the inhibitory peptide zVAD-fmk control generation of ceramide. Whether proteases directly affect PC-PLC or acidic sphingomyelinase activities remains to be investigated. One consequence of ceramide production would be a loss in mitochondrial membrane potential as suggested by the major decrease of $\Delta\Psi_m$ in cells treated by exogenous ceramides (Fig. 6). We described above that anti-Fas and anti-HLA class I mAbs partially reduced $\Delta\Psi_m$. The decrease of $\Delta\Psi_m$ is inhibited by zVAD-fmk but not by Ac-DEVD-CHO and Ac-YVAD-CHO, suggesting that DEVD- and YVAD-specific proteases act downstream of mitochondrial events. Cytochrome *c* was recently reported to be released from mitochondria in apoptotic cells, to activate DEVD-specific caspases and induce apoptotic effects in cell-free systems containing cytosol (55, 56). However, in these cell-free assays, release of cytochrome *c* from the mitochondria was not accompanied by changes in $\Delta\Psi_m$. In Jurkat cells undergoing apoptosis after Fas ligation, a loss of cytochrome *c* function was also reported (57). Interestingly this cytochome *c* inactivation was accompanied by a slow and partial decrease of $\Delta\Psi_m$ and inhibited by zVAD-fmk peptide. Therefore the loss of cytochrome *c* activity during Fas-mediated apoptosis is likely to involve a step dependent on a zVAD-specific protease. Altogether these results support a close relationship between caspases and ceramide in the signaling of apoptosis mediated by Fas and HLA class I molecules.

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