Rhodamine 110-Linked Amino Acids and Peptides as Substrates To Measure Caspase Activity upon Apoptosis Induction in Intact Cells†

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ABSTRACT: Caspases (cysteine aspartate-specific proteases) are a structurally related group of cysteine proteases that cleave peptide bonds following specific recognition sequences. They play a central role in activating apoptosis of vertebrate cells. To measure apoptosis induced by various stimuli and at an early apoptotic stage, caspases are an ideal target. This is especially the case when apoptotic cells have to be analyzed ex vivo before phagocytes remove them. A new and sensitive caspase assay is based on a substrate that contains only aspartate residues linked to rhodamine 110. With this and similar substrates, we are able to detect intracellular caspase activation by flow cytometry after apoptosis induction in intact hematopoetic cell lines.

The activation of caspases is a hallmark of apoptosis (*1*). Caspases comprise a structurally related group of cysteine proteases that show specificity for cleaving peptide bonds following aspartate residues (*2*). In mammals, at least 14 different caspases have been described (*3*). All caspases are synthesized as latent zymogens (procaspases) which are processed either autocatalytically or by other caspases at internal cleavage sites. The active enzyme acts as a heterotetramer composed of two small and two large subunits (*2*).

According to their function and primary sequence, caspases are divided into two subclasses. Caspases-8 and -10 contain tandem repeats of the so-called "death effector domain" (DED),[1] and caspases-1, -2, -4, -5, and -9 contain a distinct but analogous "caspase recruitment domain" (CARD) (*2*). Both types of domains recruit caspases to their activator complexes, and these caspases are called initiator, signaling, or class I caspases (*4*). DED-containing caspases are activated through DED-containing death receptor associated proteins.

Many apoptotic stimuli lead to the release of cytochrome *c* from mitochondria. Binding of cytochrome *c* and dATP to Apaf-1 in the cytoplasm enables Apaf-1 to associate with its CARD domain with the CARD domain of procaspase-9 (*4*), thereby triggering the activation of a caspase-9-initiated proteolytic cascade (*5*).

The central death machinery of apoptosis, where the initiated caspase cascades converge, consists of the effector or executioner caspases (class II caspases), with caspase-3 as the best characterized member. Activation of effector caspases requires proteolytic processing by upstream initiator caspases. The substrates of caspase-3 are considered to ensure apoptosis and to be responsible for the characteristic morphology that occurs during apoptosis (*6*). In most of the caspase-3 substrates, the recognition site is DEVD (the single-letter code for amino acids is used), where cleavage occurs after the second D (*7*).

It is difficult to detect apoptosis in vivo and ex vivo since the apoptotic cells are removed by cells with phagocytic activity. The activation of caspases is one of the earliest marker for apoptosis. It probably occurs before the cell undergoing apoptosis is recognized by phagocytes. The externalization of phosphatidylserine to the outer leaflet of the plasma membrane, which can be detected by annexin V binding, requires the activation of caspases (*8*, *9*). Therefore, caspases are an ideal early target for measuring apoptosis. Activation of caspases is an irreversible event since proteolytically inactivated molecules can be renewed only by their de novo synthesis.

We have identified a class of new substrates capable for the measurement of caspase activity. The core of the substrates is rhodamine 110. The amino groups of rhodamine 110 are coupled to amino acids or peptides. The nonfluorescent bis-substituted peptide derivatives of rhodamine 110 are intracellularly cleaved to green fluorescent monosubstituted rhodamine 110 and free rhodamine 110 (*10*). Rhodamine 110 fluorescence is measured upon excitation with 488 nm laser light. Since these substrates are cell-permeable, caspase activity is measured in intact cells by flow cytometry.

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[1] Abbreviations: Apaf-1, apoptosis protease activating factor-1; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; DED, death effector domain; CARD, caspase recruitment domain; PARP, poly-(ADP-ribose) polymerase; D_2R, $(Asp)_2$-rhodamine 110; dD_2R, (dextra-$Asp)_2$-rhodamine 110; DDT, dithiothreitol; $(DEVD)_2R$, $(AspGluValAsp)_2$-rhodamine 110; E_2R, $(Glu)_2$-rhodamine 110; N_2R, $(Asn)_2$-rhodamine 110; z-VAD-fmk, *N*-benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture. Jurkat, CEM, Molt 4, SKW 6.4, and Mono Mac 6 cell lines were maintained in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal calf serum (Biochrome), L-glutamine (Gibco), and penicillin/streptomycin (Gibco). To the Mono Mac 6 cell cultures 1 mM pyruvate was added (Gibco).

Peptide Synthesis. The rhodamine 110 (R)-labeled peptides, D_2R, E_2R, N_2R, dD_2R, and $(DEVD)_2R$, were synthesized by Interactiva Biotechnologie GmbH (Ulm, Germany). All rhodamine 110-coupled peptides were at least 95% pure. The stock solution was 50 mM in DMSO/ethanol at a 1:1 ratio.

Induction of CD95-Mediated Apoptosis. CD95 receptor stimulation was performed with the monoclonal antibody anti-APO-1 (*11*) at a concentration of 1 μg/mL in the presence of 10 ng/mL protein A as cross-linker if not stated otherwise.

Caspase Assay. CD95-mediated apoptosis was induced in 1 mL (10^6 cells/mL) of cells for the indicated time intervals. 2-Mercaptoethanol was added to a final concentration of 10 mM. The rhodamine-labeled peptide substrates were then added to a final concentration of 50 μM if not stated otherwise. Osmotic shock treatment was done by adding 1 mL of H_2O, followed by incubation for 10 min at 37 °C. The osmotic shock was stopped by adding 0.1 mL of ice-cold 10 × PBS. Cells were then washed in 1 × PBS and resuspended in 300 μL of 1 × PBS for FACS analysis. The cellular rhodamine 110 green fluorescence (515−545 nm) was measured with excitation by a 488 nm argon laser on a FACScan flow cytometer (Becton Dickinson) (*10*). A total of 10^4 cells per sample were analyzed with the CellQest software. Where mentioned, osmotic shock treatment was omitted. In the absence of osmotic shock, the substrates were added to a final concentration of 50 μM. The cells were then incubated for 10 min at 37 °C and used directly for FACS measurement.

PhiPhiLux Caspase Assay. PhiPhiLux staining was carried out as described by the manufacturer (OncoImmunin Inc., Maryland; catalog number A304R6G-3). Briefly, after induction of apoptosis cells were washed with ice-cold 1 × PBS. Cell pellets were gently resuspended and incubated in RPMI 1640 (Gibco) containing 10% fetal calf serum and 9 μM PhiPhiLux at 37 °C for 1 h in the dark. Then the cells were diluted in 500 μL of ice-cold flow cytometry dilution buffer (provided by the kit) and measured immediately by using a FACS Calibur (Becton Dickinson) in channel FL2.

Annexin V and D_2R Costaining. Cells were seeded in 24-well plates at a concentration of 10^6 cells/mL in a 1 mL volume. Apoptosis was induced by addition of a final concentration of 4 μM staurosporine (Sigma). At various time intervals, cells were harvested and split into two equal aliquots. One aliquot was stained with annexin V−FITC (Pharmingen) according to the manufacturer's protocol; the other was stained with D_2R (see above) and analyzed by flow cytometry (FACScan, Becton Dickinson). The green fluorescence of annexin V positive cells was detected in the FL1 channel, the intracellular fluorescence of rhodamine 110 in the FL2 channel.

Inhibition of Caspases by z-VAD-fmk (N-Benzoylcarbonyl-Val-Ala-Asp-fluoromethyl Ketone). A 10 mM stock solution of z-VAD-fmk (Enzyme Systems Products) was prepared in DMSO. z-VAD-fmk was added to a final concentration of 10 μM 30 min prior to the induction of apoptosis.

Western Analysis and PARP Cleavage. In 10^7 cells (10^6 cells/mL) CD95-mediated apoptosis was induced for the indicated time intervals. Cells were washed 2 times with 1 × PBS and resuspended in 40 μL of lysis buffer [20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.1 mM EGTA, 350 mM NaCl, 1% Igepal CA630 (Sigma), 0.5 mM dithiothreitol (DTT), 20% glycerol, 0.1 mM (4-amidinophenyl)methanesulfonyl fluoride (APMSF) (Boehringer-Mannheim), 1 μg/mL pepstatin A (Sigma), 1 μg/mL leupeptin (Sigma), 2 mM *o*-phenanthroline (Serva), 1 μg/mL aprotinin (Sigma)]. The protein concentration was determined by the BCA-Pierce-Protein Assay kit according to the protocol of the manufacturer. For Western analysis, 50 (or 100) μg of protein was separated on 10−20% gradient gels (Daiichi) and blotted onto nitrocellulose membranes (Amersham) according to standard protocols. The monoclonal anti-caspase-3 antibody was purchased from Transduction Laboratories (clone 19, C31720), the monoclonal anti-caspase-8 antibody from Pharmingen (clone B9-2, 66231A), the polyclonal anti-PARP antibody from Boehringer-Mannheim (1835238), and the monoclonal anti-β-actin antibody AC-15 from Sigma. Secondary antibodies were anti-mouse or anti-rabbit IgG1-coupled antibodies to horseradish peroxidase (Santa Cruz Biotechnology) at a dilution of 1:5000. To reuse membranes, they were stripped for 5 min in 0.2 M NaOH at room temperature.

RESULTS

The human T leucemic cell lines Jurkat and CEM have been treated with monoclonal antibodies against CD95 in the presence of protein A to induce apoptosis. Before or just when apoptotic alterations appeared under the light microscope, cells were harvested, incubated with caspase substrates, and analyzed by flow cytometry for caspase activation by $(Asp)_2$-rhodamine 110 (D_2R) and related substrates.

To test the specificity of caspase activation, we used *N*-benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), a caspase peptide inhibitor including caspases-1, -3, -4, and -7 (references in Calbiochem signal transduction catalog & technical resource 1999). In Jurkat cells, which were treated only with z-VAD-fmk, 1 and 3 h with the anti-APO-1 antibody and protein A, or 3 h with the anti-APO-1 antibody and protein A in the presence of z-VAD-fmk, no increase in fluorescence occurred (Figure 1A). When the cells were treated as in Figure 1A but the caspase substrate D_2R was added after the induction of apoptosis, a weak increase of fluorescence was observed after 1 h and a strong increase after 3 h of apoptosis induction (Figure 1B). This increase could be completely inhibited by the addition of z-VAD-fmk, which is a known inhibitor of caspases-1, -3, -4, and -7 (Figure 1B). As a negative control substrate, we used $(dextra\text{-}Asp)_2$-rhodamine 110 (dD_2R) where the D-enantiomer of aspartic acid is bound to rhodamine 110. Unexpectedly, we found a shift in fluorescence at 1 and 3 h after apoptosis induction (Figure 1C). This shift was weaker as compared to D_2R as substrate and was also inhibitable by z-VAD-fmk (Figure 1C). Thus, dD_2R seems to be a substrate for caspases since an increase in fluorescence does not appear without

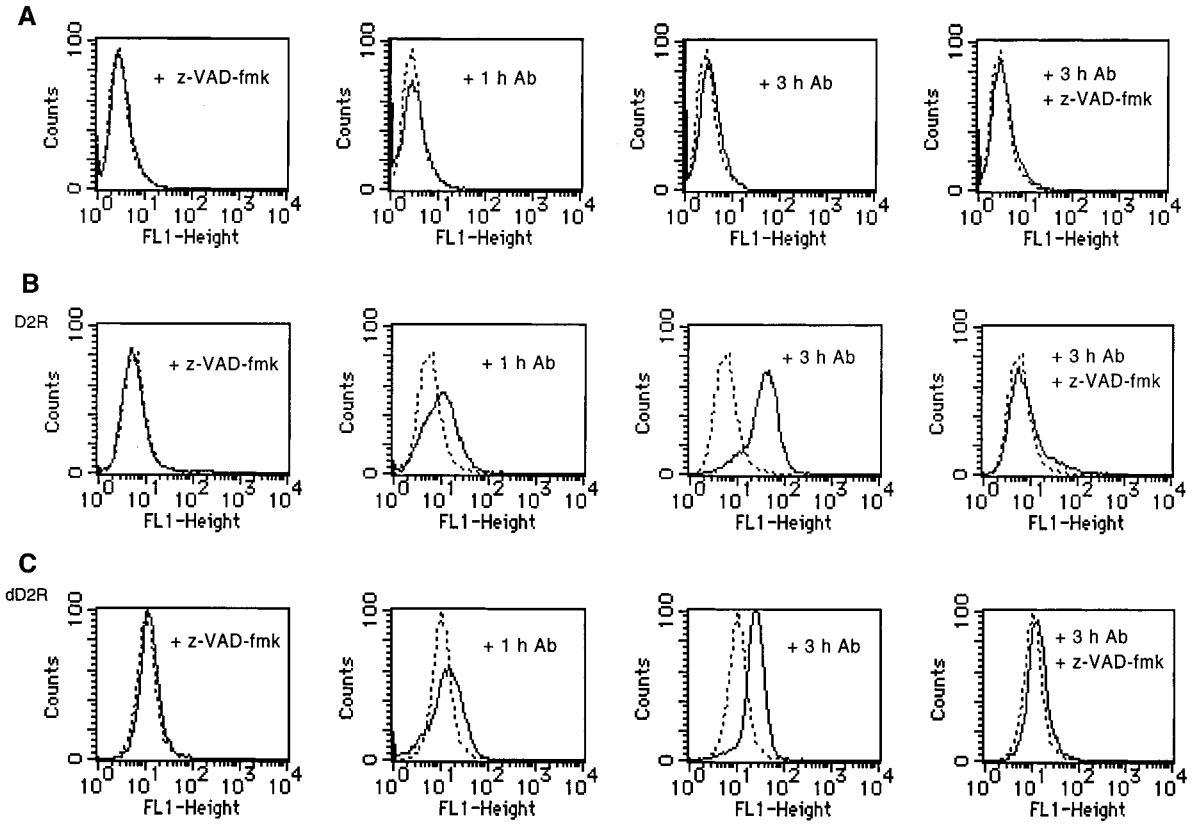

FIGURE 1: Detection of caspase activity in Jurkat cells. Cells were treated with z-VAD-fmk alone, or apotosis was activated with anti-APO-1 antibody and protein A in the absence or presence of z-VAD-fmk. The dotted line is untreated Jurkat cells. The solid line corresponds to treatment with z-VAD-fmk alone, 1 h anti-APO-1 treatment, 3 h anti-APO-1 treatment, or 3 h anti-APO-1 treatment in the presence of z-VAD-fmk as indicated. (A) Incubation without fluorogenic caspase substrate. (B) Incubation with the fluorogenic caspase substrate D_2R. (C) Incubation with the fluorogenic control substrate dD_2R. Labeled cells were analyzed by FACS. Ab, anti-APO-1 antibody.

induction of apoptosis (dotted line in Figure 1C) and z-VAD-fmk inhibits the cleavage of dD2R.

Similar data were obtained using CEM cells (data not shown). At 3.5 h of apoptosis induction, a clear shift in fluorescence was detected. But this shift was not as strong as compared to Jurkat cells. After 3.5 h, however, most of the CEM cells were already dying. The fluorescence shift was inhibited by z-VAD-fmk (data not shown). Using dD_2R as substrate, no clear shift in fluorescence could be observed (data not shown). The differences of CEM cells compared to Jurkat cells could be due to a weaker activation of caspases or to the activation of a different subset of caspases.

Cleavage of the caspase-3-specific sequence $(AspGluValAsp)_2$-rhodamine 110 [$(DEVD)_2R$] was significantly weaker as compared to D_2R in Jurkat cells after 3 h of apoptosis activation (Figure 2). However, in CEM cells there was no significant difference observed with either D_2R or $(DEVD)_2R$ as caspase substrate (Figure 2). This could mean that the main caspase which becomes activated in CEM cells after activation of the CD95 pathway is caspase-3. In Jurkat cells, in addition to caspase-3, other caspases become activated after induction of CD95-mediated apoptosis. Activation of caspase-3, -8 and -9 in both cell systems, Jurkat and CEM, has been described (*12*).

We also have tested $(Glu)_2$-rhodamine 110 (E_2R) and $(Asn)_2$-rhodamine 110 (N_2R) as caspase substrates. Both yielded an increase in fluorescence using the conditions shown in Figures 1 and 2 (data not shown). As shown for D_2R and $(DEVD)_2R$, cleavage occurs only after activation

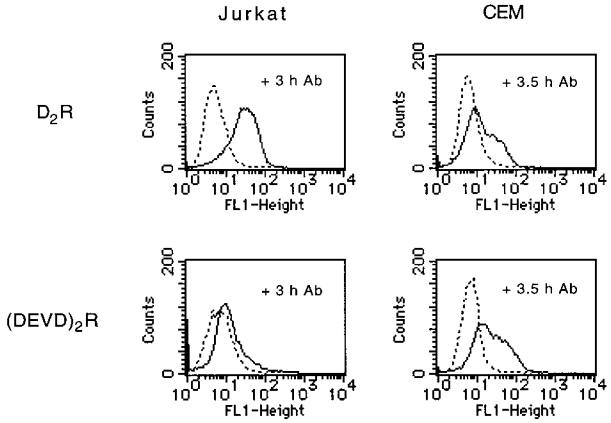

FIGURE 2: Comparison of the caspase substrate D_2R with the caspase-3 substrate $(DEVD)_2R$. In Jurkat (left) or CEM (right) cells, apoptosis was induced with anti-APO-1 antibody and protein A for the time indicated. Cells were then stained with the caspase substrate D_2R (upper diagrams) or $DEVD_2R$ (lower diagrams) and analyzed by FACS. Ab, anti-APO-1 antibody.

of apoptosis, and it is completely inhibited by treatment with z-VAD-fmk (data not shown). Possible explanations are that asparagine and glutamic acid when bound to rhodamine 110 are recognized by a caspase or several caspases or that during the course of apoptosis other proteases are activated. These proteases are likely to act downstream of caspases since their activation is inhibitable by z-VAD-fmk.

Activation of caspase-3 after CD95 triggering in Jurkat and CEM cells has been described (*12*). We confirmed cleavage of the caspase-3 substrate PARP under our experi-

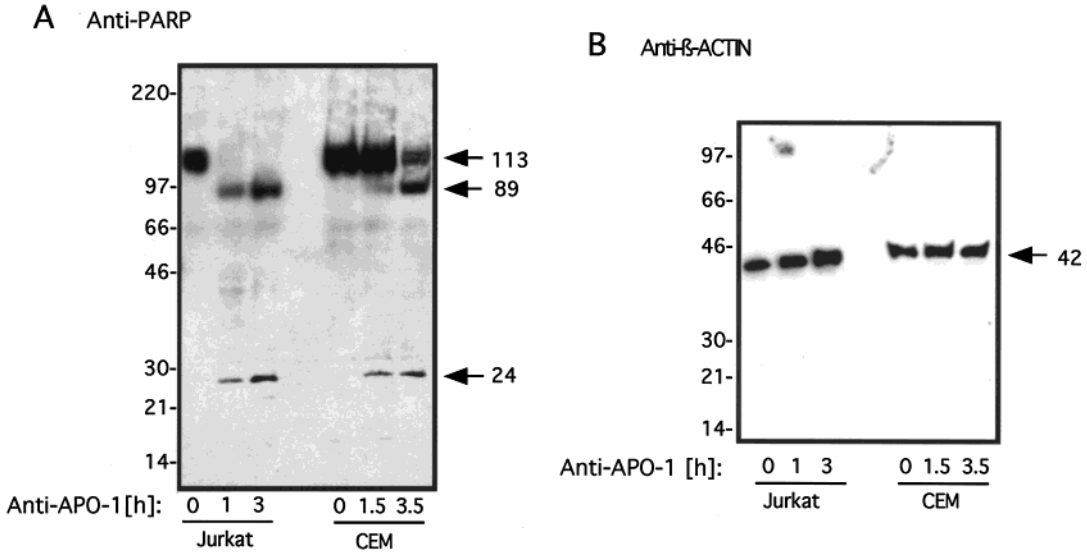

FIGURE 3: PARP cleavage of anti-CD95-treated Jurkat and CEM cells. Jurkat and CEM cells were treated with 1 μg/mL anti-CD95 antibody and 10 ng/mL protein A for the indicated time points. Extracts were prepared, and Western analysis was performed as described under Experimental Procedures. (A) Anti-PARP antibody (exposure time: 1.5 min). (B) Anti-β-actin antibody (exposure time: 1 min). The protein size markers (in kDa) are given on the left side. Arrows at the right side point to the expected protein or protein fragment sizes in kDa.

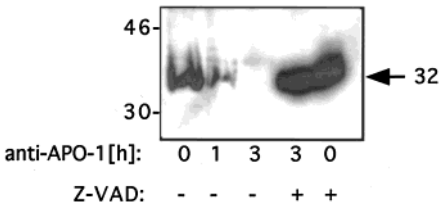

FIGURE 4: Western analysis of procaspase-3 cleavage and inhibition by z-VAD-fmk. Jurkat cells were treated with 1 μg/mL anti-CD95 antibody and 10 ng/mL protein A for 0, 1, and 3 h in the presence or absence of z-VAD-fmk as indicated. Extracts were prepared, and Western analysis with an anti-caspase-3 antibody was performed as described under Experimental Procedures (exposure time: 10 min). The protein size markers (in kDa) are given on the left side. The arrow at the right side points to procaspase-3 (in kDa). Z-VAD, z-VAD-fmk.

mental conditions (Figure 3A). The disappearance of the 113 kDa PARP signal and the occurrence of the 89 and 24 kDa signals started in the case of Jurkat cells after about 1 h; in the case of CEM cells after about 3 h. Equal amounts of protein loading were shown by β-actin staining (Figure 3B). PARP cleavage was inhibited by the addition of z-VAD-fmk (data not shown). Caspase-3 and caspase-8 cleavage occurred at approximately the same time points as PARP cleavage (Figure 4 and data not shown). In Jurkat cells, the inactive 32 kDa procaspase-3 disappeared 3 h after CD95 activation. The originating cleavage products p17 and p12 of caspase-3 are not recognized by the antibody. Cleavage of procaspase-3 was inhibited in the presence of z-VAD-fmk (Figure 4). In the presence of z-VAD-fmk, the procaspase-3 signal appeared stronger compared to the control lane without induction of apoptosis and without the addition of z-VAD-fmk. Equal protein loading was confirmed by reprobing the filter with a monoclonal antibody to β-actin (not shown). Similar results with delayed kinetics were observed for CEM cells (data not shown). Taken together, cleavage of caspases-3 and -8 and PARP cleavage correlated with the data obtained by measuring the activation of caspases with D_2R.

To show that rhodamine 110-linked caspase substrates become cleaved with other inducers of apoptosis, Jurkat cells were treated with staurosporine. Caspase activation was measured with D_2R and $(DEVD)_2R$ as substrates. Using D_2R as substrate after 18 h treatment with 1 μM staurosporine in the absence of osmotic shock, a clear shift in fluorescence was detected (data not shown). However, this shift was only partially inhibited by the addition of z-VAD-fmk (data not shown). With $(DEVD)_2R$, no shift in fluorescence could be observed with Jurkat cells under the same conditions (data not shown).

To demonstrate the applicability of our caspase assay in more detail, we compared D_2R staining with annexin V staining (Figure 5A) and PhiPhiLux staining (Figure 5B). By treating the acute lymphoblastic human T cell line Molt 4 with 4 μM staurosporine, the percentage of D_2R-positive cells is higher compared to annexin V staining (Figure 5A). However, this difference was less pronounced by using 1 μM staurosporine (data not shown). But there was a clear correlation between annexin V positive cells and cells cleaving D_2R. Staining with propidium iodide showed that most of the cells were dead after 2.5 h of incubation with staurosporine (data not shown). As seen in Figure 5A dead cells are stained by annexin V−FITC but not by D_2R.

Comparison of our D_2R caspase assay with the PhiPhiLux caspase assay showed a lower background by using D_2R (Figure 5B). After the onset of apoptosis, the fluorescence signal due to cleavage of D_2R was significantly higher than the signal due to cleavage of PhiPhiLux. It was also shown that in addition to T cell lines D_2R cleavage occurred also in the human B cell line SKW 6.4 and the human monoblastic cell line Mono Mac 6 after induction of apoptosis.

DISCUSSION

D_2R is the first reliable substrate allowing the measurement of caspase activity in intact cells. Induction of programmed cell death leads to a significant increase in the fluorescence intensity of D_2R-loaded cells. This event can be blocked by

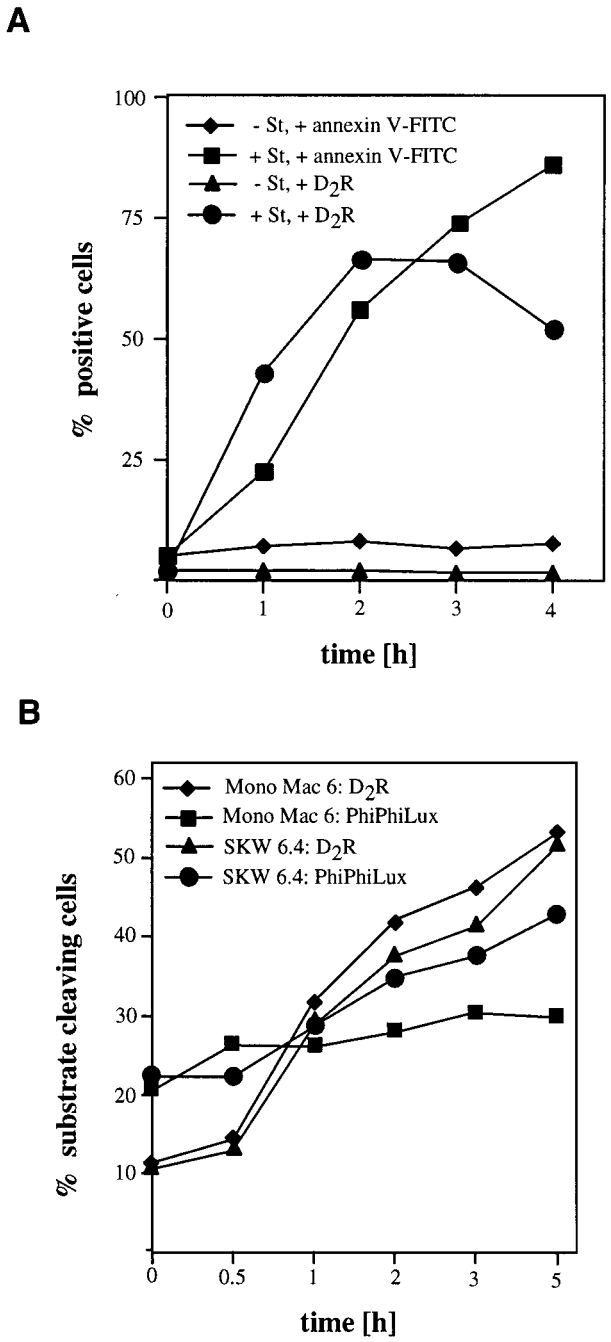

FIGURE 5: D_2R caspase assay compared to annexin V−FITC and PhiPhiLux staining. (A) Apoptosis was induced in Molt-4 cells by addition of 4 μM staurosporine. Cells were incubated for the indicated time period and stained either with annexin V−FITC or with D_2R. A representative experiment out of three performed is shown. (B) SKW 6.4 and Mono Mac 6 cells were treated with 1 μg/mL anti-CD95 antibody (here from Cell Diagnostica, Münster, Germany) for the indicated time period. For efficient induction of CD95-mediated apoptosis, 4 μg/mL cycloheximide was added simultaneously to Mono Mac 6 cells. Cells were then stained either with D_2R or with PhiPhiLux. The standard deviations (not shown) did not exceed 12% for D_2R and 16% for PhiPhiLux from three independent experiments. In this experiment, the final concentration of D_2R was 150 μM. St, staurosporine.

the broad-spectrum caspase inhibitor z-VAD-fmk. Compared to other described caspase assays, our method is capable of detecting simultaneously a variety of different caspase family members upon its activation, which increases its sensitivity. Measuring more than one activated caspase family member results in stronger signals, which is important if only a few cells are available for the assay (e.g., in clinical samples). Substrates such as PhiPhilux, which are also used to measure caspase activation via FACS (*13*, *14*), poorly penetrate the cell membrane and need a longer incubation time. This may be the reason why D_2R staining results in lower background and higher sensitivity than PhiPhiLux staining. With PhiPhiLux staining, it is not possible to measure apoptotic events shorter than 1 h (the staining time).

Assays based on coumarin-labeled peptide substrates (e.g., Clontech) are cell membrane impermeable. Therefore, they work only in cellular extracts. Our substrate can be used not only for the measurement of caspase activity in cell extracts, but also in flow cytometry. This makes it possible to monitor caspase activity, e.g., in various subpopulations of cells defined by other markers in a mixed cell population.

To have a reliable assay for bringing all the different caspase substrates into the cells, we used a protocol that included an osmotic shock. But osmotic shock treatment can be omitted. In cases where we have directly compared the D_2R caspase assay with and without osmotic shock, we observed no significant difference. Therefore, D_2R penetrates the cell membrane.

Part of our work was dedicated to increase the specificity of our assay, thus making it capable of detecting a single caspase. The monitoring of caspase-3 activity appeared to be most interesting since this caspase plays an important role upon apoptosis induction in many experimental and physiological systems. Caspase-3 knockout mice show a severe phenotype with early lethality due to failure of developmental programmed cell death of neuronal cell precursors, which argues for the central role of caspase-3 at least in neurons (*15*). Numerous groups described important roles of caspase-3 in various experimental systems (*16*, *17*). The substrate sequence DEVD is specific for caspases-3 and -7 (*7*). By using $(DEVD)_2R$ as a substrate in our assay system, we observed a much weaker fluorescence increase as compared with D_2R in Jurkat cells. Since we have shown activity of caspase-3 in Jurkat and CEM cells, D_2R must also be the substrate of caspases other than caspase-3 or -7 in Jurkat cells. In addition, $(DEVD)_2R$ substrate is significantly bigger and also carries more charged groups on the surface. Therefore, penetration of the plasma membrane is probably not as efficient as with D_2R.

In another series of experiments, we have tested our substrates in the human T cell line Molt-4, induced to undergo apoptosis with staurosporine. Here, in contrast to Jurkat cells, the measurement of caspase activation with $(DEVD)_2D$ resulted in a stronger increase in fluorescence as with D_2R. This could be due to a different caspase activation pattern by staurosporine, different membrane permeability upon blockage of cellular kinases, different membrane lipid composition, or the presence of different caspases in Molt-4 cells compared to Jurkat cells.

In Jurkat cells, caspase activation induced by staurosporine could not be completely inhibited by z-VAD-fmk. Compared to activation of CD95-mediated apoptosis, which occurs after a few hours, staurosporine-induced cell death started after 18 h. Very likely, these inducers activate different caspases showing a different inhibitory profile toward z-VAD-fmk. But we cannot exclude that D_2R is also a substrate for an enzyme that does not belong to the caspase family.

For a better control of our experimental system, we have also tested E_2R and N_2R in our assays since they are similar to D_2R. These substances also became cleaved after induction of CD95. The cleavage was inhibited by the presence of z-VAD-fmk. In these experiments, N_2R resulted in a similar shift in fluorescence as D_2R, E_2R in a weak shift. This indicates that proteases other than caspases may also become activated in the course of apoptosis (*18*−*20*). For control purposes, also dD_2R was applied in the assay. We have expected that caspases would not cleave substrates containing D-amino acids as it has been described for aminopeptidases (*21*) and hence thought that dD_2R could serve as a negative control. However, the cleavage of dD_2R, detected in Jurkat but not in CEM cells, shows that caspases or caspase-like enzymes are able to use rhodamine 110 linked to the D-enantiomer of aspartic acid.

The rhodamine 110-based substrates have been used previously for the activity detection of a variety of proteases (*21*, *22*). In addition, caspases have functions not only in apoptosis but also in the processing of proinflammatory cytokines. In fact, the first identified member of the caspase family, interleukin-1β converting enzyme (ICE), which is now termed caspase-1 (*23*), was originally described as the enzyme responsible for the maturation of prointerleukin-1β. Especially in T lymphocytes there may be caspases constitutively or inducibly activated to process cytokines. In our assay system, this may lead to higher background since our substrates are probably not specific for only the particular caspases which become activated during apoptosis. Knockout experiments indicate that murine caspases-1 and -11 are not critical for apoptosis (*2*). Targeted disruption of the respective caspase genes causes lack of lipopolysaccharide-induced interleukin-1α and interleukin-1β production due to lack of activation of caspase-1. Caspase-1(−/−) mice are also defective in interleukin-18 maturation; they display defective lipopolysaccharide induction of tumor necrosis factor-α, interleukin-6, and interferon-γ production. In addition, caspase-11 directly interacts with caspase-1, thus facilitating its activation at least upon induction by certain activators (*24*).

The purity of the D_2R is a critical parameter of the assay. It must be more than 95% pure; otherwise, the signal of monosubstituted rhodamine 110 causes a significant background signal which diminishes the sensitivity of the method. The assay in the current form allows measurement of caspase activity in subpopulations of cells. D_2R and similar substrates have the prerequisite to be the basis for the development of a diagnostic method to detect apoptosis ex vivo in diseases and treatments where apoptosis plays a role, e.g., to monitor the therapy success in cancer, to measure apoptosis in HIV-infected patients, or to detect caspase-1 or caspase-11 activity during inflammation.

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