Phosphatidylserine Externalization during CD95-induced Apoptosis of Cells and Cytoplasts Requires ICE/CED-3 Protease Activity*

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Phosphatidylserine (PS), a lipid normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma membrane leaflet during apoptosis to serve as a trigger for recognition of apoptotic cells by phagocytes. The mechanism of PS export during apoptosis is not known nor is it clear whether the nuclear changes that typify apoptosis contribute in any way to this event. Here, we demonstrate that ligation of the CD95 (Fas/APO-1) molecule on Jurkat cytoplasts induces dramatic PS externalization similar to that observed during apoptosis of intact cells. Apoptosis of both cells and cytoplasts was associated with proteolytic processing of CPP32, a member of the interleukin-1β converting enzyme (ICE)/CED-3 protease family, to its active form. Fodrin, a component of the cortical cytoskeleton, also underwent proteolytic cleavage during apoptosis of both cytoplasts and intact cells. Strikingly, CPP32 activation, fodrin proteolysis, and PS externalization were all inhibited in the presence of peptide inhibitors of ICE/CED-3 family proteases. These data provide strong support for the notion that the cell death machinery is extranuclear and is likely to be comprised of one or more members of the ICE/CED-3 family and that activation of this machinery does not require nuclear participation.

Apoptosis is a mode of cell death known to be under molecular control and is central to numerous physiological and pathological processes where cells are eliminated (1–3). Although the most prominent morphological changes that occur during apoptosis typically involve the cell nucleus, studies using cyto-plasts have demonstrated that the nucleus is not required for the dramatic membrane blebbing events and the subsequent loss of viability that occurs during this process (4–6). However, it is unclear whether enucleated cells are truly apoptotic, that is, whether they can be recognized as such by macrophages and other phagocytes, as apoptotic cells are known to be (7, 8). It is formally possible that although cytoplasts undergo plasma membrane blebbing and other gross features of apoptosis, they may not acquire the membrane changes that would stimulate uptake of these cells by phagocytes.

Two major mechanisms appear to exist for the recognition of apoptotic cells by phagocytes; one mediated by a receptor that engages PS† exported to the plasma membrane during apoptosis and the other mediated by a vitronectin receptor/CD36/thrombospondin interaction that sees an as yet unidentified ligand on the apoptotic cell (9, 10). These two mechanisms appear to be mutually exclusive because macrophages that express the PS receptor do not appear to be capable of utilizing the vitronectin receptor-associated pathway (11). Thus far, the molecular events underlying these plasma membrane changes remain unknown.

Recent evidence indicates that members of the emerging ICE/CED-3 family of proteases may occupy a critical position in the cellular apparatus that effects the destructive changes within the cell during apoptosis (see Refs. 12–14 for recent reviews). However, it is unclear whether these proteases become activated without any nuclear participation and whether the plasma membrane changes that stimulate uptake of apoptotic cells, such as PS externalization, requires the activity of these proteases.

Recently, several groups have reported that PS externalization during apoptosis can be sensitively measured using annexin V, a protein with a natural high affinity for this aminophospholipid. Using this method it has been found that PS export to the outer leaflet of the plasma membrane is an early and widespread event during apoptosis of cells from numerous lineages and is inhibited by overexpression of apoptosis repres-sor proteins such as Bcl-2 and v-Abl (15, 16). Here, we demonstrate that PS externalization also occurs during apoptosis of Jurkat cytoplasts in response to Fas (CD95) ligation with a similar kinetics to that observed during Fas-induced apoptosis of intact cells. We also show that CPP32 (also called Yama or Apopain), a member of the ICE/CED-3 protease family, becomes activated during Fas-induced apoptosis of both cells and cytoplasts. In line with these observations, we demonstrate that fodrin, a cytoskeletal protein known to undergo proteolysis during apoptosis, is also cleaved during apoptosis of cytoplasts. Strikingly, all of these events were blocked in the presence of peptide inhibitors of ICE/CED-3 family proteases. These data provide further strong support for the notion that the cell death machinery is largely extranuclear and also suggest a causal link between ICE/CED-3 protease activation and the membrane changes that occur during apoptosis.

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The abbreviations used are: PS, phosphatidylserine; ICE, interleukin-1β converting enzyme; VAD-FK, Val-Ala-Asp-fluoromethylketone; DEVD-FK, Asp-Glu-Val-Asp-fluoromethylketone; YVAD-Ck, Tyr-Val-Ala-Asp-chloromethylketone; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibody to a-fodrin (nonerythroid spectrin) was purchased from Chemicon International; anti-b-actin monoclonal antibody was purchased from ICN. Anti-CPP32 monoclonal was purchased from Transduction Laboratories, and anti-Fas mAb was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). TVAD-CK peptide was purchased from Calbiochem (San Diego, CA). Annexin V-FITC was purchased from Biowhittaker Inc and Clontech Inc. (Palo Alto, CA; ApoAlert Kit). Ficoll-400 and all other reagents were purchased from Sigma.

**Cell Culture and Induction of Apoptosis**—Jurkat cells were cultured in RPMI 1640/5% fetal calf serum under standard conditions. To initiate apoptosis, cells or cytoplasts were incubated with anti-Fas IgM antibody (clone CH-11) at concentrations ranging from 25 ng to 400 ng/ml. Apoptosis was quantitated by monitoring changes in cell size and granularity by flow cytometry and by staining with annexin V-FITC, which detects phosphatidylserine externalization on the outer leaflet of the plasma membrane (15, 16).

**Enucleation of Jurkat Cells**—Cytoplasts were prepared from Jurkat cells by density centrifugation using a previously described procedure with modifications (17). Exponentially growing cells were incubated at 37°C for 1 h at a density of 0.7 x 10^7 cells/ml in the presence of 21 μM cytochalasin B, to depolymerize microtubules, followed by layering over discontinuous Ficoll gradients and centrifugation for 1 h at 80,000 x g. Gradients were prepared by sequentially layering the following Ficoll-400 solutions (dissolved in Hanks’ balanced salt solution/10% fetal calf serum) in a round-bottomed centrifuge tube; 25 (6 ml), 17 (6 ml), 16 (3 ml), 15 (3 ml), and 12.5% (6 ml). Before use, gradients were equilibrated by incubation at 37°C for 1 h in an atmosphere of 5% CO2. Cells were applied to the gradients in a volume of 3 ml, followed by overlaying with 5 ml of Hanks’ balanced salt solution. Cytoplasts were collected from the interface between the 12.5 and 15% layers and were washed twice in complete medium before use. Cytoplasts were routinely inspected for purity by staining with a 10 μM solution of Hoechst 33342 in formalin, followed by evaluation under the fluorescence microscope (see Fig. 1).

**Electrophoresis and Western Blotting**—Proteins were separated under reducing conditions for 2 h at 70 V in SDS-polyacrylamide gels as described previously (18). Separated proteins were then blotted onto polyvinylidene difluoride membrane at 100 mA for 2 h. Blots were blocked for 1 h in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dried milk and then probed for 2 h with an appropriate dilution of the primary antibody diluted in the same buffer and washed for 1 h in several changes of TBST, followed by probing for a further 1 h with appropriate peroxidase-coupled secondary antibodies (Amersham Corp.). Bound antibody was detected by enhanced chemiluminescence (Pierce).

**RESULTS AND DISCUSSION**

Previous studies have demonstrated that several features of apoptosis, including plasma membrane blebbing, cytoplasmic vacuolation, and ultimately, loss of plasma membrane permeability, can be induced in cytoplasts derived from several cell types by exposure to several pro-apoptotic stimuli (4–6). Because recognition of apoptotic cells by phagocytes is a critical event in the programmed cell death process (7), we wished to determine whether apoptotic cytoplasts also acquired one of the known membrane changes that trigger phagocytosis of apoptotic cells: externalization of phosphatidylserine on the outer leaflet of the plasma membrane (9, 11, 15). Thus we prepared cytoplasts by enucleating Jurkat T lymphoblastoid cells and explored the capacity of these to die in response to ligation of CD95 (Fas/Apo-1), as compared with normal Jurkat cells.

Fig. 1 illustrates that both Jurkat cells and cytoplasts underwent dramatic cell surface blebbing after exposure to anti-CD95 IgM (CH-11) as reported in previous studies (5, 6), suggesting that apoptosis was occurring under these conditions. We then examined whether cytoplasts also externalized PS in response to CD95 ligation, as would be expected if the mechanism responsible for PS export during apoptosis remained intact in enucleated cells. Using recombinant annexin V, a protein that has a high affinity for PS, we found that Jurkat cytoplasts did indeed exhibit PS externalization in response to...
CD95 ligation, to a remarkably similar degree to that seen in Jurkat cells exposed to the same stimulus (Fig. 2). As previously reported (15, 16), apoptosis-associated PS externalization was detected well before cells or cytoplasts lost membrane integrity (as assessed by trypan blue exclusion). Typically, loss of membrane integrity followed 8–12 h after PS was detected on the outer leaflet of the PM. These data demonstrate that the machinery responsible for PS export to the outer plasma membrane leaflet during apoptosis operates independent of any nuclear events that take place during the cell death process.

Many recent studies have implicated proteases, particularly those of the ICE/CED-3 family, as important participants in the sequence of events that result in the dismantling of the cell during apoptosis (see Refs. 12–14 for recent reviews). Many proteins have been found to undergo proteolysis during apoptosis, although practically all of these substrates are localized within the nucleus (19–21). One of the few non-nuclear substrates that has been reported to date, fodrin (nonerythroid spectrin), is a major component of the plasma membrane-associated cytoskeleton and has been found to undergo proteolysis in response to numerous pro-apoptotic stimuli, including CD95 ligation (18, 20, 22). Therefore, it was of considerable interest to determine whether fodrin was also targeted for proteolysis in the absence of a nucleus. Thus, we probed lysates from untreated versus anti-CD95-treated cytoplasts for fodrin and found that this protein was cleaved into its characteristic 150- and 120-kDa cleavage products during apoptosis of cytoplasts (Fig. 3A). This demonstrates that the protease responsible for apoptosis-associated fodrin proteolysis is also activated in cytoplasts.

Although it has not been formally shown that apoptosis-associated fodrin proteolysis is mediated by an ICE/CED-3 family protease, we have recently found that neutralization of CPP32 activity in a cell-free model of apoptosis also results in loss of fodrin-cleaving activity (20), suggesting that fodrin cleavage during apoptosis is mediated by CPP32 or a downstream protease that is activated as a consequence of CPP32 activation. Therefore we asked whether CPP32 was activated during CD95-mediated apoptosis of Jurkat cytoplasts. Fig. 3 (B

**Fig. 3.** Proteolysis of fodrin and processing of CPP32 occur in the absence of a nucleus. Jurkat cytoplasts were either left untreated or were induced to undergo apoptosis by exposure to anti-Fas mAb (400 ng/ml), and cell lysates were made at the indicated times. A, degradation of fodrin occurs in response to Fas-ligation in cytoplasts, whereas actin remains intact under the same conditions. Some spontaneous fodrin proteolysis is also observed in untreated cytoplasts as a consequence of spontaneous apoptosis. Actin was used as a protein loading control. B, CPP32 undergoes proteolytic processing from the 36-kDa pro-form during apoptosis of both Jurkat cells and cytoplasts. Only the 17-kDa chain of the mature p17/p12 heterodimer is detectable with the antibody used. The blot shown has been deliberately overexposed to enhance detection of the 17-kDa chain, which is not as efficiently recognized as the pro-form. C, time course of CPP32 processing during Fas-induced apoptosis of Jurkat cytoplasts.
and C) demonstrates that proCPP32 underwent processing to its active heterodimer form during apoptosis of cytoplasts, as has also been recently reported for intact cells (23, 24).

Because apoptosis in intact cells can be prevented by specific peptide inhibitors of ICE/CED-3 family proteases, we explored whether cytoplast apoptosis (as assessed by PS externalization, fodrin proteolysis, and CPP32 maturation) could also be blocked by these inhibitors. Strikingly, we found that apoptosis-associated PS externalization in cytoplasts was strongly inhibited in the presence of all ICE family inhibitors tested (VAD-FK, DEVD-FK, and YVAD-CCK; Fig. 4). Interestingly, CD95-initiated apoptosis in intact cells was also strongly inhibited by both VAD-FK and DEVD-FK but to a lesser extent with YVAD-CCK. VAD-FK also inhibited both fodrin proteolysis and CPP32 maturation under the same conditions (Fig. 5, A and B).

We have found that cytoplasts behave similarly to intact cells with respect to all features of apoptosis studied. These data, coupled with previous studies (4–6), suggest that all aspects of apoptosis described (12–14). Although it is likely that one or more of these proteases are key mediators of apoptosis, it remains unclear as to how these proteases become activated during apoptosis and what their important substrates are. Many of the substrates for ICE/CED-3 family proteases that have been described to date are localized to the nucleus (19, 20, 21, 25). Our observations that CPP32 becomes activated during apoptosis of cytoplasts rules out the possibility that activation of this protease requires a nuclear factor or that cleavage of a nuclear substrate (such as poly(ADP-ribose) polymerase, lamin B, the 70-kDa U1 small nuclear ribonucleoprotein, and DNA-dependent protein kinase) is necessary for cytosolic events in apoptosis. Of course, these observations do not rule out the possibility that cleavage of nuclear substrates may serve to delay or accelerate apoptosis under certain conditions. Finally, our observations with VAD-FK also indicate that there is likely to be one or more ICE/CED-3 proteases that are activated upstream of CPP32 upon ligation of Fas, because this inhibitor blocked apoptosis upstream of the CPP32 processing step (Fig. 5). Recent reports suggest that at least one of the proteases upstream of CPP32 in the CD95-initiated death cascade is MACH/FLICE (26, 27).

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Fig. 5. VAD-FK inhibits both fodrin proteolysis and CPP32 processing in cells and cytoplasts during CD95-mediated apoptosis. Cells or cytoplasts were exposed to the indicated treatments for 4 h, and lysates were made and subsequently probed for either fodrin (A) or CPP32 (B).