

Temporal Phases in Apoptosis Defined by the Actions of Src Homology 2 Domains, Ceramide, Bcl-2, Interleukin-1 β Converting Enzyme Family Proteases, and a Dense Membrane Fraction

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Abstract. We have begun to explore the mechanisms of apoptosis using a cell-free system based on extracts from *Xenopus* eggs. Nuclei assembled or placed in these extracts undergo the morphological changes typical of apoptosis and eventually disintegrate. We used this system to investigate the potential involvement in apoptosis of proteins containing Src homology 2 (SH2) domains, which are known to interact with specific tyrosine-phosphorylated ligands. SH2 domains from a number of signaling proteins, including Lck, Src, and Abl, inhibited apoptosis when present at concentrations of 10–100 nM. The inhibition was dependent on specific interaction with endogenous tyrosine-phosphor-

ylated ligands. A synthetic peptide ligand for Src family SH2 domains also inhibited apoptosis in a phosphotyrosine-dependent manner. Kinetic analysis defined three phases in the apoptotic process occurring in this cell-free system. SH2 domains and ceramide act throughout the first 60–90 min of the process (the “initiation” phase). Next, Bcl-2, interleukin-1 β converting enzyme family (CPP32-like) proteases, and the heavy membrane fraction act in a period occurring ~90–120 min after the start of incubation (the “sentencing” phase). In the final phase (“execution”), the process of active nuclear destruction ensues.

APOPTOSIS is a normal physiological process of cell death observed in multicellular organisms (for reviews see 7, 34, 37, 42, 45, 46). The apoptotic death pathway can be initiated by a variety of stimuli, including DNA damage, the withdrawal of growth factors, and the binding of certain ligands to cell surface receptors. Although the death stimulus and cellular context can vary widely, it is thought that apoptosis involves a conserved biochemical machinery. Supporting this notion of a common pathway are several observations: first, apoptotic cells undergo a stereotypical sequence of morphological changes, including plasma membrane blebbing, chromatin condensation, and the shrinkage and fragmentation of the nucleus and cytoplasm, resulting in the formation of cell fragments termed “apoptotic bodies.” Second, endonucleases and proteases are usually activated, causing the fragmentation of genomic DNA and the cleavage of certain proteins such as fodrin (25), nuclear lamins (19, 33), and poly (ADP-ribose) polymerase (16, 20, 31, 40). Fi-

nally, there are a number of proteins whose function in regulating apoptosis is conserved phylogenetically. For example, the human Bcl-2 protein can inhibit apoptosis when expressed in the cells of a variety of species, including the nematode, *Caenorhabditis elegans* (e.g., 15, 32, 37, 44, 45). Bcl-2 belongs to a family of proteins (for reviews see 32, 37). Some members of this family, e.g., Bcl-2, Bcl-x_L, *C. elegans* Ced-9, and the adenovirus E1B 19-kD protein, are able to inhibit apoptosis. Others, such as Bax, Bad, Bcl-x_S, and Bak (1, 9, 17), have the opposite effect of promoting cell death. Thus, cell survival may be regulated in part by the ratio of Bcl-2-type to Bax-type molecules (38, 47, 49).

Also showing conserved function in apoptosis is a large family of cysteine proteases related to the *C. elegans* protein, Ced-3 (50), and the vertebrate protein, interleukin-1 β converting enzyme (ICE)¹ (26). Most recently, a particular subfamily of these enzymes, consisting of Ced-3, CPP-32/Yama/Apopain (5, 11, 30, 39, 43), MCH2 (12), and ICE-LAP3/CMH-1/MCH3 (6, 13, 21), has been suggested to play a central part in the apoptotic process.

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; HM, heavy membrane; ICE, interleukin-1 β converting enzyme; PLC, phospholipase C; PTyr, phosphotyrosine; SH2, Src homology 2.

The precise roles of these molecules in the death pathway, as well as the identities of other molecular participants in the process, remain to be elucidated. We recently described a novel cell-free system that may help to unravel aspects of apoptotic biochemistry and the function of Bcl-2 (29). This system is based on extracts from eggs of the South African clawed frog, *Xenopus laevis*. *Xenopus* egg extracts have been an important tool for studying cellular processes such as nuclear membrane assembly, nuclear protein import, DNA replication, and cell cycle regulation (for review see 28). We found that it was also possible to prepare "apoptotic" extracts that mimic the nuclear shrinkage, chromatin condensation, and DNA fragmentation observed in cells dying by apoptosis (29). Apoptotic activity in these extracts requires the presence of a heavy membrane (HM) fraction consisting mostly of mitochondria and is inhibited by the addition of baculovirus-expressed Bcl-2 protein. Moreover, we found that apoptosis in this system involves a latent phase, lasting ~90–120 min, that occurs whether or not nuclei are present. Protection by Bcl-2 occurs only if Bcl-2 is present during this latent period; thus, it would appear that the antiapoptotic role of Bcl-2 is due primarily to its interaction with cytoplasmic, rather than nuclear, targets.

Apoptosis in the *Xenopus* system is not affected by inhibitors of tyrosine phosphorylation such as herbimycin A (29) and genistein (see Results). Thus, protein tyrosine kinases are apparently not involved in the portion of the cell death pathway at work in the cell-free system. However, free phosphotyrosine (PTyr; 10–20 mM) was found to have a marked inhibitory activity (29). This suggested that protein tyrosine phosphatases or other molecules interacting with tyrosine-phosphorylated proteins might be important for apoptosis in this system. We were thus led to explore the possibility that Src Homology 2 (SH2) domains are involved in the apoptotic pathway. SH2 domains (for reviews see 2, 36) are a type of modular sequence element often found in signaling molecules. These domains are known to interact with specific protein sequences containing phosphorylated tyrosine residues. The binding of SH2 domains to their ligands provides a means for the cell to assemble protein complexes in a manner readily controlled by tyrosine kinases and phosphatases. We now show that the SH2 domains from certain signaling proteins, when added to the *Xenopus* cell-free system in the form of glutathione-S-transferase (GST) fusion proteins, can inhibit apoptosis. Moreover, apoptosis in this system is inhibited by a synthetic peptide known to interact with the SH2 domains of Src family kinases; this effect is dependent on tyrosine phosphorylation of the ligand peptide.

The data support a role of SH2–PTyr interactions in the apoptotic pathway. Moreover, our investigations show that the events controlled by SH2 domain interactions, ceramide, Bcl-2, CPP32-like proteases, and the HM fraction define two distinct temporal phases in the apoptotic process. These phases precede a third stage, during which the nuclei are actively destroyed.

Materials and Methods

The preparation, fractionation, and use of *Xenopus* egg extracts were as

described (28, 29). In experiments with unfractionated extracts, 50 μ l of extract was mixed with ATP-regenerating system (consisting of 2.5 μ l of a 200 mM solution of phosphocreatine, 1 μ l of a 100 mM solution of ATP, pH 7.0, and 1.5 μ l of a solution of creatine phosphokinase (5 mg/ml; Sigma Chemical Co., St. Louis). Then, 1–3 μ l of a suspension of rat liver nuclei (at $\sim 10^6$ nuclei per μ l) was added, as well as 1 μ l of rhodamine-labeled nuclear import substrate (TRITC-HSA-NLS [a TRITC-labeled conjugate of human serum albumin with a synthetic nuclear localization signal peptide]) to monitor nuclear intactness, as described (28). GST–SH2 domain fusions and synthetic peptides were then added from stock solutions at 20–50 times the final concentration. HM-depleted extracts were reconstituted from 45 μ l of egg cytosol and 5 μ l of light membrane suspension obtained from M-phase extracts, as described (28).

The expression and purification of GST fusion proteins were as described (4). Briefly stated, the GST–SH2 domains of Lck were produced by PCR amplification of the desired region of wild-type (Y192), F192, and E192 Lck constructs (amino acids 121–224), using the proofreading Vent DNA polymerase (New England Biolabs, Beverly, MA). The PCR fragments were inserted in frame into the pGEX-3T prokaryotic expression vector (14), and the resulting GST–SH2 fusion proteins were purified by glutathione–Sepharose affinity chromatography. The fusion proteins were dialyzed (using a microdialysis apparatus [Pierce Chemical Co., Rockford, IL]) against a buffer containing Hepes (20 mM) and KCl (50 mM) before use. Synthetic peptides were obtained from Research Genetics (Huntsville, AL).

Phosphorylation of the synthetic peptide (RNLNNGGFYIAPR) comprising the site surrounding Y192 in Lck was assayed essentially as described previously (27): peptide (4 mg/ml), γ -[32 P]ATP (1 mCi/ml), and *Xenopus* egg extract (7.4% vol/vol, corresponding to a protein concentration of ~ 2.6 mg/ml) were incubated at 22°C for the times indicated, in 25 μ l of a buffer containing 50 mM Hepes/K, pH 7.4, 10 mM MgCl₂, 5 mM MnCl₂, and 1.2 mM Na₃VO₄. The reaction was stopped with the addition of 155 μ l of 3.2% TCA and 20 μ l of a solution of BSA (10 mg/ml). After 30 min on ice, the samples were centrifuged, and 100 μ l of each supernatant was applied to a 2.5-cm circle of phosphocellulose membrane (Whatman P-81). The filters were washed seven to eight times in 75 mM *o*-phosphoric acid in 50-ml conical tubes. After drying, the filters were placed in 4 ml of scintillation fluid and the radioactivity was counted. All the results presented here were qualitatively reproducible. However, the time at which

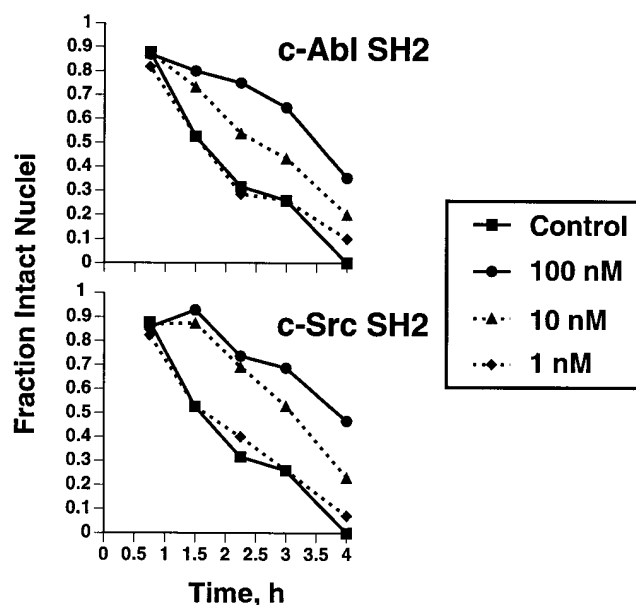


Figure 1. SH2 domains from Abl and Src inhibit apoptosis in the cell-free system. GST fusions of the SH2 domains of c-Abl and c-Src were added, at the indicated concentrations, to an apoptotic egg extract containing sperm chromatin. The fraction of intact nuclei was assayed at various times thereafter, as previously described (29).

nuclear destruction began to occur varied from extract to extract, making it impossible to average the results from separate experiments in a meaningful way. Each figure is representative of at least four experiments giving similar results.

Results

GST-SH2 Domain Fusions Specifically Inhibit Apoptotic Activity in the Cell-free System

To investigate whether SH2-PTyr interactions are involved in the cell-free apoptosis system, we prepared GST fusion proteins containing the SH2 domains from several signaling proteins and added them to the extracts. Fig. 1 shows that the SH2 domains from c-Src and c-Abl produced a concentration-dependent inhibition of apoptosis when present at concentrations of ≥ 10 nM. A similar effect was seen when fusions of GST with the SH2 domains of Grb-2 and Stat1 were added (not shown).

To determine whether the effects of SH2 domains added to the cell-free system were based on specific interactions with phosphorylated ligands, we took advantage of point mutations in the SH2 domain of Lck that disrupt PTyr-dependent binding. Previous studies (4) had shown that the function of the Lck SH2 domain can be regulated by modification of a particular amino acid residue, Y192. Phosphorylation at this site, which is located next to the EF loop of the SH2 domain, markedly lowers the affinity of the SH2 domain for phosphotyrosine-containing protein ligands. Mutants in which this Tyr residue was changed to Phe (F192) and Glu (E192) had been found to mimic the unphosphorylated and phosphorylated states, respectively, of Y192. We added the wild-type and mutant Lck SH2s to the *Xenopus* cell-free system. As shown in Fig. 2, the SH2 domain from the constitutively inactive Lck-E192 mutant was unable to inhibit apoptosis. On the other hand, the Lck-F192 mutant SH2, which binds constitutively to

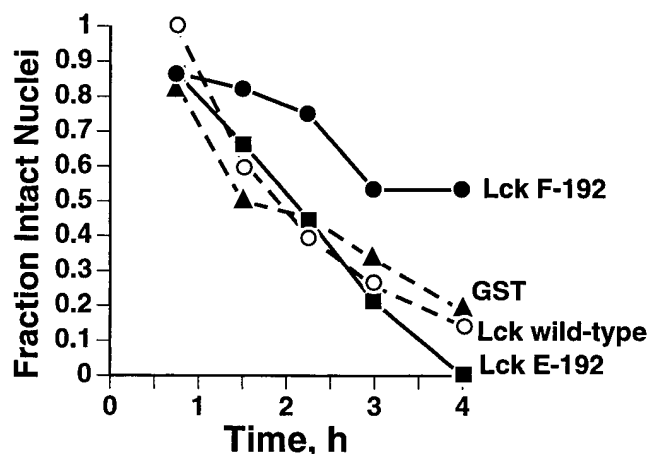


Figure 2. Effect of wild-type and mutant SH2 domains from Lck. The experiment was performed as in Fig. 1. GST-SH2 domain fusions were added at 100 nM. Note that the wild-type (Y192) Lck SH2 domain is unable to inhibit apoptosis, as is the E192 mutant, which mimics the phosphorylated form of Y192. However, the F192 mutant, which mimics the unphosphorylated form of Y192, inhibits apoptosis in the extract.

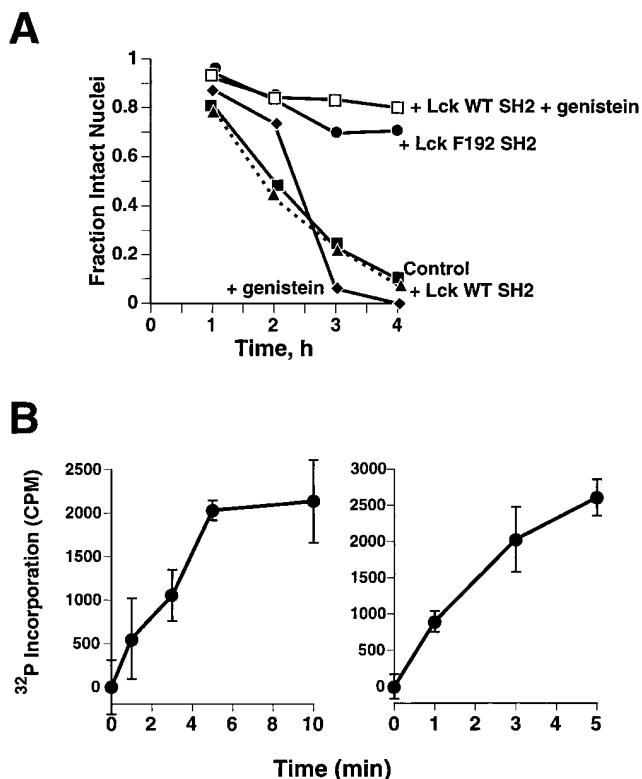


Figure 3. The wild-type Lck SH2 domain is inactive in the extract because it becomes phosphorylated. (A) Genistein, an inhibitor of tyrosine kinases, restored the ability of the wild-type Lck SH2 domain to inhibit apoptosis. The experiment was done as in Fig. 2, except that genistein (10 μ M) was added to the indicated samples either separately or along with the wild-type (WT) Lck SH2 domain-GST fusion (100 nM). (B) A kinase in the *Xenopus* egg extract can phosphorylate a synthetic peptide corresponding to the site surrounding Y192 in Lck. The peptide (RNLDNGGFY-IAPR) was incubated in a buffer containing γ - 32 P]ATP and *Xenopus* egg extract (diluted as described in Materials and Methods) for the times indicated, and the incorporated radioactivity was assayed (see Materials and Methods). Two separate experiments are shown.

phosphotyrosine-containing ligands, was as potent an inhibitor as the Src and Abl SH2 domains.

The wild-type Lck SH2 domain, like the nonbinding E192 mutant, was unable to inhibit apoptosis. This result could be explained if we supposed that the wild-type Lck SH2 domain became phosphorylated on Y192 by a kinase present in the extract and consequently had a lowered affinity for the tyrosine phosphorylated ligand, similar to the E192 mutant. This hypothesis predicted that if phosphorylation on Y192 were prevented, the wild-type SH2 domain would function like Lck-F192; i.e., it would inhibit apoptosis in the cell-free system. To test this prediction, we added the tyrosine kinase inhibitor, genistein, either separately or with the wild-type Lck SH2 domain. Genistein, added by itself, had only a slight effect on the kinetics of nuclear destruction in the apoptotic cell-free system (Figs. 3 and 4 B; this suggests that any critical tyrosine phosphorylation events involving proteins endogenous to the extract had already occurred before the addition of genistein). How-

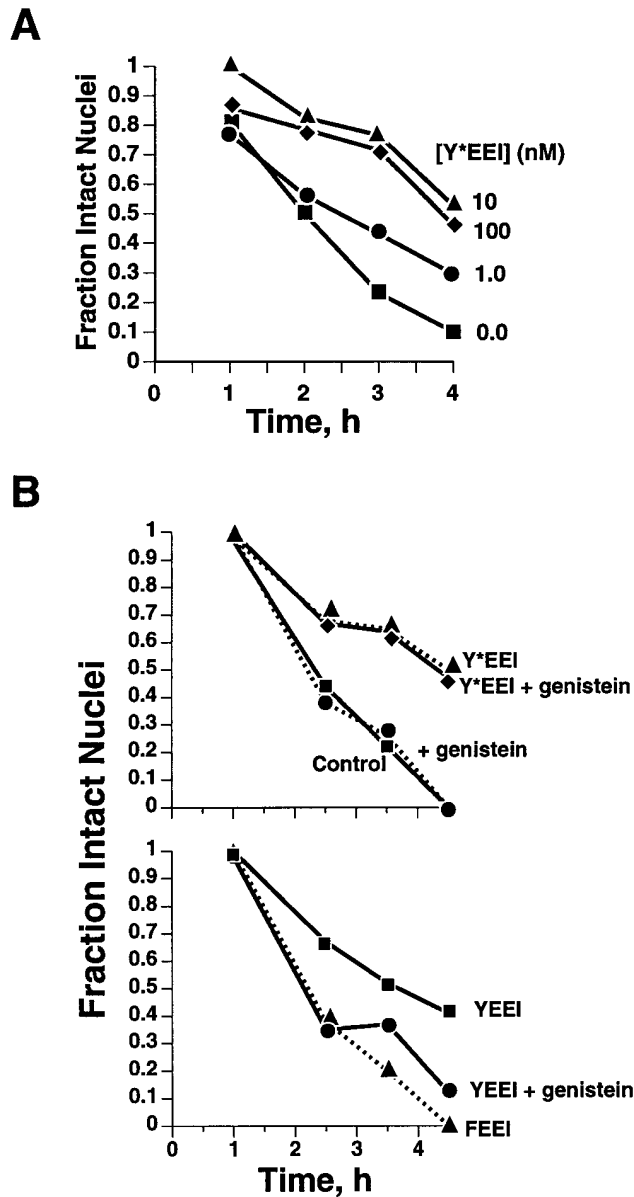


Figure 4. A synthetic peptide ligand for Src family SH2 domains, EPQY*EEIPIYLK (Y*EEI), inhibits apoptosis in the *Xenopus* cell-free system in a PTyr-dependent manner. (A) The indicated concentrations of phosphopeptide were added at the start of incubation. (B) (Top) The phosphopeptide (denoted by Y*EEI) was added to the extract at a final concentration of 5 nM, in the presence or absence of genistein. Genistein has no effect on its activity. (Bottom) The unphosphorylated form of this peptide (YEEI) and an unphosphorylatable mutant peptide (FEEI) were added at 5 nM. Note that the FEEI peptide has no inhibitory activity. The unphosphorylated peptide (YEEI) inhibits apoptosis nearly as well as the Y*EEI, but this inhibition is reversed in the presence of genistein. Thus, the peptide is apparently phosphorylated by a kinase present in the extract.

ever, the coaddition of genistein and the wild-type Lck SH2 domain produced a marked inhibitory effect, similar to that of the F192 mutant (Fig. 3 A). Therefore, the Lck SH2 domain is likely to be phosphorylated on Y192 by an endoge-

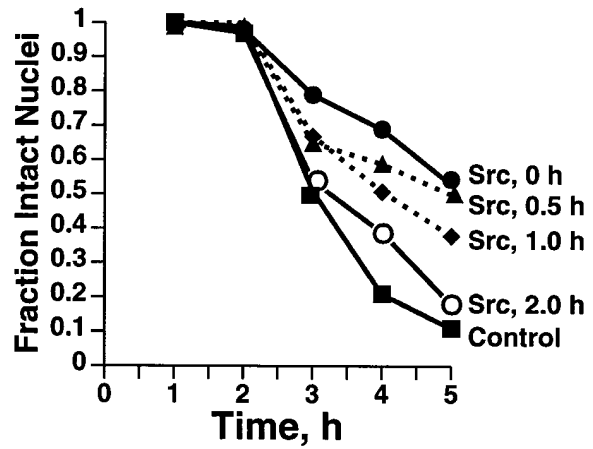


Figure 5. The Src SH2 domain inhibits an activity important in the early portion of the apoptotic process. The experiment was performed as above, except that the GST-Src SH2 fusion was added (final concentration 100 nM) at the indicated times after the start of incubation.

nous protein tyrosine kinase in the *Xenopus* egg extracts, and thus inactivated. To examine this more directly, we assayed the phosphorylation of a synthetic peptide corresponding to the sequence surrounding Y192. Fig. 3 B shows that this peptide is phosphorylated by the extract in a time-dependent manner; the specific activity was estimated at 7.7 ± 0.2 pmol per min per mg of extract protein. Thus, a kinase is present in the extract that is able to phosphorylate this site in the Lck SH2 domain. We conclude from the experiments shown in Figs. 1–3 that the effects of the exogenous SH2 domains are dependent on specific interactions with endogenous tyrosine-phosphorylated ligands.

To confirm that PTyr-SH2 domain interactions are important for apoptosis in the cell-free system, we examined the effects of adding an exogenous synthetic phosphopeptide, EPQY*EEIPIYLK (abbreviated Y*EEI; Y* represents PTyr), known to interact with Src family SH2 domains (41). Fig. 4 A shows that this peptide was able to inhibit apoptosis even at concentrations as low as 1 nM. In control experiments (Fig. 4 B), we found that a nonphosphorylatable mutant peptide, EPQFEEIPIYLK (FEEI), was ineffective, arguing again that the inhibitory effect is dependent on the specific interaction of SH2 domains with sequences containing PTyr residues. The effect of adding the unphosphorylated peptide (YEEI) was similar to that seen with Y*EEI, suggesting that YEEI can be phosphorylated by kinases endogenous to the extract (Fig. 4 B). In support of this idea, we found that the protein tyrosine kinase inhibitor, genistein, was able to reverse the inhibitory effect of YEEI (Fig. 4 B). To probe the specificity of these effects further, we tested another synthetic phosphopeptide, corresponding to the SH2 domain-binding region surrounding residue Y1021 of phospholipase-C γ 1 (DNDY*IIPDPK; 35). This peptide had no significant effect on the apoptotic process in the cell-free system, even at concentrations as high as 1 μ M (see Fig. A1). This is additional evidence that interactions involving specific SH2 domains are important in the *Xenopus* system.

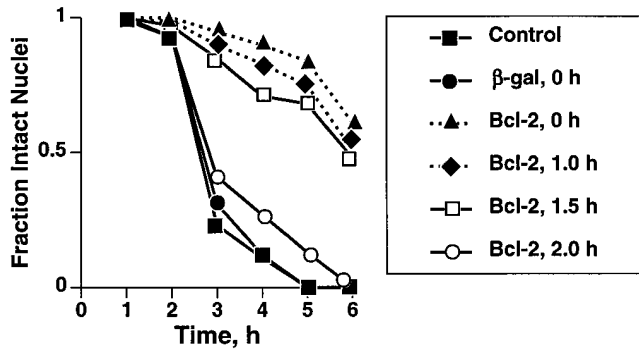


Figure 6. The Bcl-2 protein acts later in the apoptotic process, after ~ 1.5 h of incubation. Lysates from SF9 cells (0.01 vol, as described; 29) infected with Bcl-2 baculovirus or, as a control, β -galactosidase baculovirus were added at the indicated times after the start of incubation. Note that Bcl-2 is much less effective when its addition is delayed at least ~ 2 h after the start of incubation.

SH2 Domains Interact in the Early Phase of the Apoptotic Process

To determine when in the apoptotic process PTyr-SH2 interactions are important, we performed experiments in which the Src SH2 domain-GST fusion was added to the cell-free system at various times after the start of incubation (Fig. 5). As before (Fig. 1), the Src SH2 domain slowed the kinetics of nuclear destruction markedly when added at the start of incubation. However, when the addition of this SH2 domain was delayed by intervals ranging from 30–120 min, its inhibitory activity was gradually reduced. These results demonstrate that the SH2 domain interactions are important throughout the initial portion of the apoptotic process.

Bcl-2 and the Heavy Membrane Fraction Act Later Than SH2 Domains in the Apoptotic Process

Previously, we showed that Bcl-2 can inhibit apoptosis by acting during the early cytoplasmic phase of the apoptotic process (29). To define more precisely the period during which Bcl-2 acts, we performed experiments similar to those described above. Bcl-2 was added at various intervals after the start of incubation, and the time course of nuclear survival was determined. Unlike the SH2 domains, Bcl-2 inhibited apoptosis effectively even if its addition was delayed up to ~ 1.5 h (Fig. 6). Thus, it would appear that the events controlled by Bcl-2 occur later in the apoptotic process than those regulated by SH2 domain-PTyr interactions.

Our previous studies also identified a requirement for an HM fraction that was enriched in mitochondria but also contained other membrane material (29). Extracts depleted of heavy membranes were found to lack apoptotic activity, and the addition of HMs restored this activity in a dose-dependent manner. To determine when the HM fraction is required during the time course of the apoptotic process occurring in the cell-free system, we performed an experiment in which the HM fraction was added at various times after the start of incubation. Fig. 7 shows that delaying the addition of the HM fraction by as much as 1.5 h

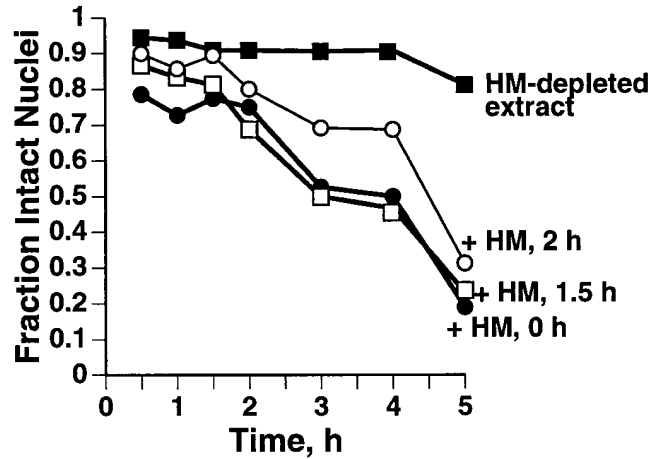


Figure 7. The heavy membrane (HM) fraction is required relatively late in the apoptotic process. Percoll-enriched HMs (29) were added at the indicated times. Note that only when the HMs were added at least 2 h after the start of incubation was there any significant delay in the kinetics of nuclear destruction.

had essentially no effect on the kinetics of nuclear destruction in the apoptotic extract. Only when the HM fraction was added at 2 h after the start of incubation was the time course of nuclear destruction delayed. We conclude that, like Bcl-2, the heavy membranes are required at a relatively late stage in the apoptotic process.

We also showed previously that a soluble ceramide analog could substitute for the HM fraction in the cell-free apoptotic system (24). This finding raised the possibility that the function of HMs was simply to produce ceramide. However, as Fig. 8 shows, ceramide acts earlier in the apoptotic process than the HMs. Like the GST-SH2 domain fusions, ceramide was most active when added at the start of incubation, and its potency declined gradually as the time of its addition was delayed. Thus, it is unlikely that the function of HMs is merely to produce ceramide.

Previous studies from many laboratories have demonstrated the involvement of cysteine proteases from the Ced-3/ICE family in the apoptotic process (see Introduc-

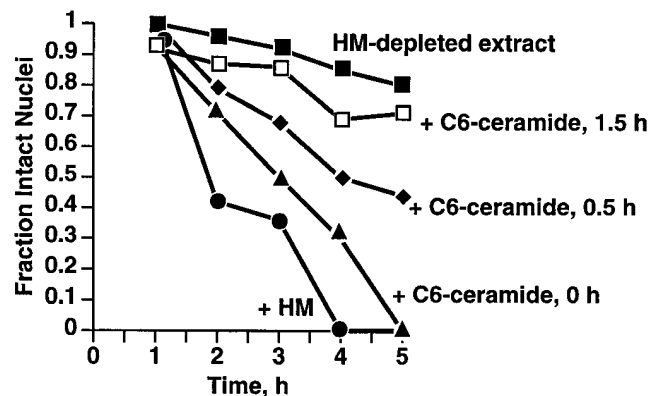


Figure 8. Ceramide acts early in the cell-free apoptotic process. A progressive loss of activity occurs when the addition of ceramide (150 μ M) is delayed 0.5–1 h.

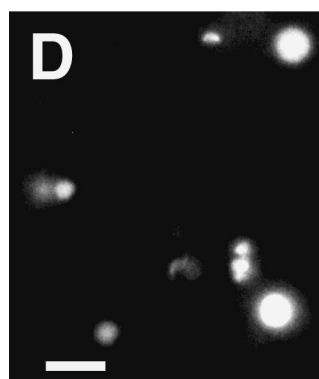
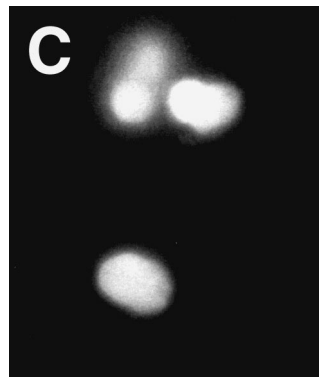
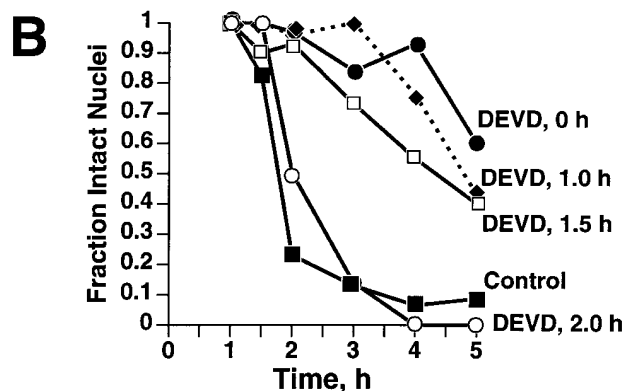
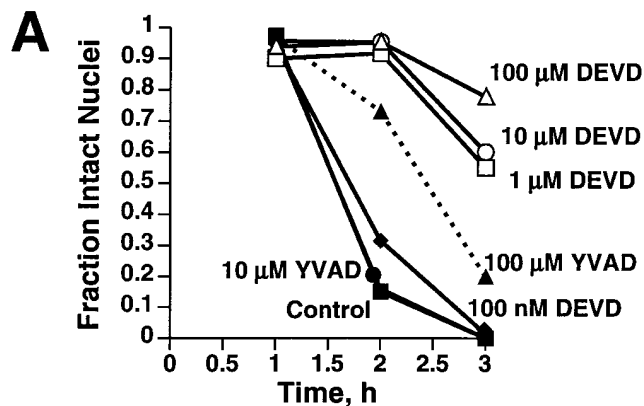


Figure 9. CPP32-like protease activity is required for apoptosis in the *Xenopus* system. The crucial proteolytic event occurs ~ 1.5 –2 h after the start of incubation. (A) The tetrapeptide inhibitors, Ac-DEVD-CHO (DEVD) and Ac-YVAD-CHO (YVAD), were added to the extract from the start of incubation at the concentrations indicated. Nuclear survival was quantitated at various times. (B) The experiment was performed as above, except that DEVD (100 μ M) was added at the indicated times. Note that the inhibitor lost its effect only when added after ~ 2 h of incubation. (C and D) Fluorescence photomicrographs of rat liver nuclei placed in apoptotic extract, incubated for 3 h in the presence (C) or absence (D) of 10 μ M DEVD. Bar, 10 μ M.

tion). To determine whether such proteases are involved in the *Xenopus* system, we tested the effects of aldehyde-based tetrapeptide derivatives that have been shown to be specific inhibitors of ICE family proteases. The compound Ac-DEVD-CHO (DEVD) has been shown to inhibit CPP32-like proteases preferentially, while Ac-YVAD-CHO (YVAD) is more selective for ICE and its close relatives (30). Fig. 9 A shows that micromolar concentrations of DEVD block apoptosis in the *Xenopus* system. YVAD also inhibited apoptosis in this system, but concentrations ~ 500 -fold higher were required. These results are similar to those described by Nicholson et al. (1995) (30) for mammalian cells, and argue that a CPP32-like, rather than an ICE-like, protease activity is required for apoptosis in the *Xenopus* cell-free system. However, because these compounds can inhibit multiple caspases, it is possible that several such enzymes are involved in this system. After this manuscript was submitted, other studies showed directly that CPP32-like caspases are activated in *Xenopus* egg extracts (3, 18).

Using the same approach as described above, we investigated the effect of delaying the addition of DEVD. The results (Fig. 9 B) showed that the protease activity was required relatively late in the apoptotic process, near the end of the 1.5–2-h latent phase. In this experiment, when Ac-DEVD-CHO was added as late as 1 h after the start of the incubation, almost no reduction in its inhibitory activity was seen, and only a partial loss of activity was observed when the inhibitor was added after 1.5 h of incubation. In contrast, the inhibitor had no activity when added after 2 h.

Discussion

A Role for SH2 Domains in Apoptosis

In this report, we have shown that apoptosis in the *Xenopus* cell-free system is inhibited by the addition of SH2 domains from several signaling proteins (Figs. 1–3 and 5). Our results with the Lck SH2 domain (Figs. 2 and 3) demonstrate that the effect is specifically due to interaction with tyrosine-phosphorylated ligands, in that it is abrogated by phosphorylation on residue Y192 of Lck; phosphorylation at this site was shown elsewhere to disrupt binding of the SH2 domain to tyrosine-phosphorylated ligands (4). Similarly, the inhibition produced by the ligand peptide, YEEI, was dependent on phosphorylation of the tyrosine residue required for interaction with SH2 domains. Thus, our results show specificity in the sense that they reflect the PTyr-dependent binding of SH2 domains and their ligands. On the other hand, the exogenous SH2 domains appear to be acting in a generic manner, inasmuch as the SH2 domains from a number of proteins can block apoptosis in this system when present at concentrations of at least 10 nM. Our results therefore do not identify which particular SH2-containing protein(s) and corresponding tyrosine-phosphorylated ligand(s) are involved in the cell-free apoptosis system.

The synthetic phosphopeptide Y*EEI inhibited apoptosis in the extract at lower concentrations than those required for the GST-SH2 domain fusions. The reason for this is unclear. Possibly these reagents act in different

ways, e.g., by interacting in a regulatory manner with specific molecules. However, we consider it likely that these molecules act by disrupting PTyr-dependent interactions between proteins endogenous to the extract. What could be the function of these endogenous proteins? SH2 domains are frequently found in signaling molecules that regulate cell proliferation. Such molecules include tyrosine kinases, tyrosine phosphatases, and the adapter molecules (e.g., Grb2) that link these enzymes to other members of signaling cascades. Grb3-3, a naturally occurring variant of Grb2 that contains a truncated, nonfunctional SH2 domain, was found to promote apoptosis, presumably by acting as a dominant negative inhibitor of a cell survival pathway (10). Our results (Figs. 1–5) are just the opposite: i.e., they suggest that some SH2 domains can be involved in promoting cell death, not survival. We suggest that the fate of the cell may rest in the balance between apoptotic and survival pathways, both controlled in part by SH2 domain interactions. After this manuscript was submitted, it was reported that c-Crk, an adapter protein containing an SH2 domain, is required for apoptosis in *Xenopus* egg extracts, and that the SH2 domain of Crk inhibits apoptosis potently in this system (8). The activity of Crk could account for our results; however, an involvement of other proteins containing SH2 domains cannot be excluded at present.

In view of our findings that suggest a role for PTyr–SH2 domain interactions in apoptosis, it may seem surprising that genistein, an inhibitor of multiple tyrosine kinases, has little or no effect on apoptosis in the *Xenopus* system. This paradox can be resolved if we suppose either that the critical phosphorylation events have already taken place before the preparation of the extracts, or that the kinases responsible for these critical phosphorylation events are insensitive to genistein. We have also observed that pervanadate (1–10 mM), an inhibitor of protein tyrosine phosphatases, inhibits apoptosis effectively in the *Xenopus* system (unpublished data; this inhibition is not merely due to the presence of peroxide in the pervanadate preparation, as control experiments with added H₂O₂ [10 mM] showed no effect on the kinetics of apoptosis). This result may suggest that tyrosine dephosphorylation of specific proteins is required for the apoptotic process in the *Xenopus* system. However, it is also possible that pervanadate blocks apoptosis by inhibiting activities other than phosphatases.

The Relationship of Ceramide and the Heavy Membrane Fraction

The role of the heavy membrane fraction is still under investigation. Previously we reported that a soluble ceramide analog can induce apoptosis in HM-depleted extracts (24). Further experiments have shown that this effect is apparently due to the synergistic effect of ceramide and a small amount of contaminating heavy membranes still remaining in the HM-depleted extracts (Farschon, D.M., and D.D. Newmeyer, unpublished results). We note that both the production of ceramide and the molecules that respond to ceramide are reported to reside in the plasma membrane (e.g., 22). If so, to explain the activity of ceramide in HM-depleted extracts, we would need to postulate that some amount of contaminating plasma membrane-derived material is present. The HM fraction could also contain a cer-

tain amount of such membranes. Thus, we considered the possibility that the function of our HM preparation was simply to increase the amount of plasma membrane-producing ceramide. However, as Figs. 7 and 8 show, time-dependent changes occur in the cell-free system that at first diminish its responsiveness to ceramide and later make it responsive to the HM fraction. Thus, it is unlikely that the function of HMs is merely to produce ceramide. Recent studies have shown that, during apoptosis, mitochondria release cytochrome c into the cytosol, leading to the activation of CPP32-like proteases. Bcl-2, through its interactions with the outer mitochondrial membrane, blocks the efflux of cytochrome c (18a, 18, 48). Whether ceramide can directly or indirectly modulate the release of cytochrome c from mitochondria is still unknown.

Temporal Stages in Apoptosis

Apoptosis could be envisioned as occurring in three stages. The first of these, “initiation,” would refer to the events that cause entry into the common death pathway. The second stage, which might be termed “sentencing,” encompasses the intracellular events that commit the cell irreversibly to the death process. Finally, the cell enters the “execution” stage, in which effector molecules, such as particular nucleases and proteases, accomplish the overt changes associated with apoptotic cell death. Each of these hypothetical stages could itself involve a series of events. To understand the apoptosis machinery, it will be necessary to know both the identities of the molecular participants in the process and whether these molecules act in sequence or in parallel.

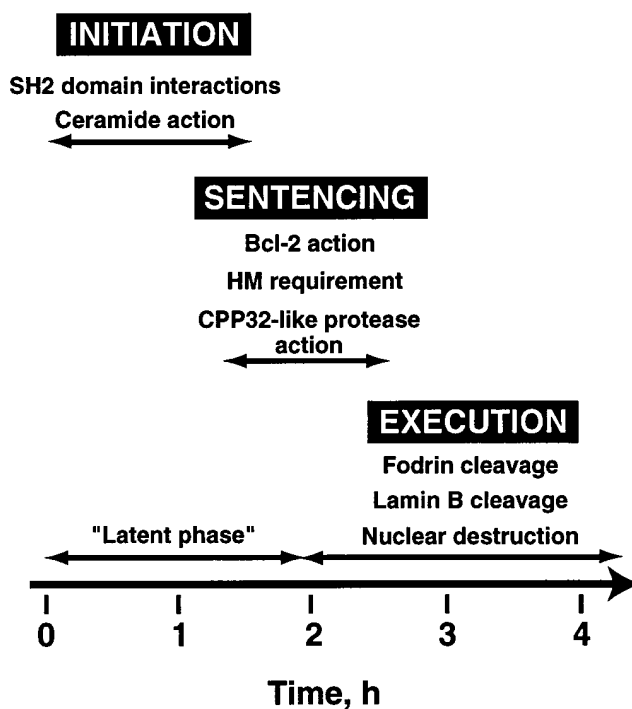


Figure 10. Summary of the approximate time intervals during which various events occur in the cell-free system.

In this report we have investigated the function of several components of the apoptotic machinery: SH2 domains, the HM fraction, ceramide, ICE family proteases, and Bcl-2, particularly with regard to the time intervals during which these reagents could affect the kinetics of nuclear destruction in the cell-free system. The events we examined fell into three main time windows, as depicted in Fig. 10. We propose that these experimentally defined time periods correspond to the three conceptual phases of apoptosis listed above.

The earliest distinguishable phase in the cell-free system, which we call "initiation," occurs in the first ~60–90 min of incubation. (Events occurring before lysis of the eggs may of course also be important for apoptosis initiation, but are outside the scope of our investigations.) The importance of SH2 domains and ceramide in this period (Figs. 5 and 8) is consistent with the expectation that these molecules would function in signal transduction. The second period, which occurs ~90–120 min after the start of incubation, is the time when Bcl-2 protein, the heavy membrane fraction, and the CPP32-related protease(s) act (Figs. 6, 7, and 9). We propose that this represents a "sentencing" phase, during which the system becomes committed irreversibly to the apoptotic pathway. Finally comes the third, or "execution" phase, beginning after ~1.5–2 h of incubation. This is the period when overt changes occur in the cell nuclei, culminating in extensive DNA fragmentation and nuclear destruction. During this phase, we also observed the cleavage of two polypeptides whose proteolysis is characteristic of apoptotic cell death (25, 33): fodrin, a cytoskeletal protein endogenous to the extract, and lamin B, present in the rat liver nuclei added to the system (18a). Our kinetics data suggest that the cleavage of fodrin and lamin B occur later than the proteolytic events inhibited by DEVD. Thus, this system appears to involve at least two distinct protease activities, which may belong to an enzymatic cascade.

It is important to note that the timing of apoptotic events in the *Xenopus* system is somewhat variable from extract to extract. This may be due, for example, to variation between animals in the proportion of eggs that are undergoing apoptosis. In extreme cases, the onset of nuclear destruction in a given extract can be as early as 45 min or as late as 4 h after incubation has begun. However, in the majority of experiments, the various events occurred within ± 30 min of the times indicated in Fig. 10. This means that, while the data in this report show that Bcl-2, the HM fraction, and DEVD-inhibitable protease activity all act in roughly the same time period, other approaches have been required to determine the sequence in which they act. Studies have now shown that mitochondria release cytochrome c into the cytosol, and that this event is blocked by Bcl-2 (18, 48); cytochrome c, when present in the cytosol, causes the activation of CPP32-related caspases (23, 18a).

The occurrence of kinetically distinguishable phases in apoptotic extracts now serves to underscore the value of this cell-free system for dissecting the crucial intracellular events involved in apoptosis. In particular, we expect this system to be useful in uncovering both the activation mechanisms and the critical substrates of the CPP32/Ced-3 family proteases.

Appendix

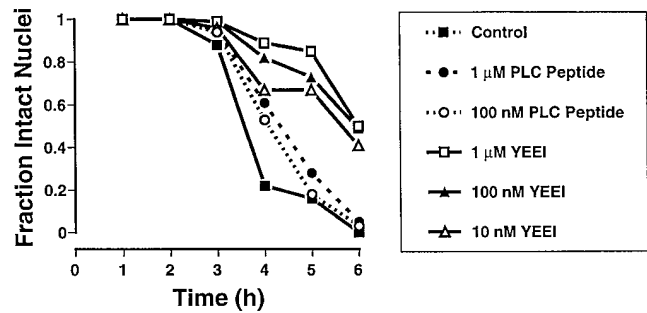


Figure A1. Comparison of SH2 ligand peptides with regard to their effects on the *Xenopus* cell-free apoptosis system. The experiment was done as in Fig. 4, except that either the YEEI peptide, or a synthetic peptide corresponding to the SH2 ligand domain of phospholipase C (PLC)- γ 1 surrounding Y1021 (PLC peptide, DNDY*IIPLDPK; 35) was added to the system at the indicated concentrations before incubation. Note that the PLC- γ 1 peptide had no significant inhibitory activity in this system.

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