Crk is required for apoptosis in *Xenopus* egg extracts

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Apoptosis is essential for the development and homeostasis of multicellular organisms. Recently, a cell-free extract prepared from *Xenopus* eggs was shown to recapitulate intracellular apoptotic pathways *in vitro*. While many stimuli have been shown to trigger apoptosis in a variety of cell types, the intracellular signaling pathways involved in apoptosis remain largely unknown. Here we show that addition of a recombinant protein containing the phosphotyrosine binding (SH2) domain from the adaptor protein crk, but not those derived from a panel of other signaling proteins, can prevent apoptosis in the *Xenopus* egg extract system. Furthermore, immunodepletion of endogenous crk protein from the egg extracts, or addition of anti-crak antisera to these extracts, prevents apoptosis. The ability to undergo apoptosis can be restored to these extracts by addition of recombinant crk protein. These results directly demonstrate that crk participates in apoptotic signaling.

Keywords: apoptosis/crk protein/signaling/*Xenopus*

Introduction

Apoptosis is a program of cellular suicide which can be initiated by a wide range of physiological stimuli. Acting in a cell-autonomous manner, the apoptotic process eliminates superfluous or harmful cells without damaging neighboring cells. Apoptosis contributes to morphogenesis and tissue formation during development, and insensitivity to apoptotic signals has been implicated in the etiology of cancer (reviewed in Ellis *et al.*, 1991; Steller, 1995). Upon receipt of apoptotic stimuli, cells undergo a latent phase of variable length during which they look normal but are condemned to die. Following this latent phase, the cells enter an execution phase which characteristically involves plasma membrane blebbing, chromatin condensation, internucleosomal cleavage of DNA, nuclear fragmentation and cell shrinkage (reviewed in Bellamy *et al.*, 1995).

A number of molecular participants in apoptotic control pathways have been identified. For example, bcl-2, originally identified as a protein whose deregulation contributes to lymphomagenesis following a chromosomal translocation, can inhibit apoptosis initiated by diverse stimuli in many different cell types (Bakhshi *et al.*, 1985; Cleary

et al., 1986; Hockenberry *et al.*, 1990; Vaux *et al.*, 1992). Competitive heterodimerization of bcl-2 and related molecules such as bad, bax, bcl-xl, bcl-xs and bak appears to play a central role in determining whether or not cells will die (Oltavi *et al.*, 1993; Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). Several proteases related to interleukin β -converting enzyme (ICE) are also required for apoptosis (reviewed in Chinnaiyan and Dixit, 1996), and in some cases well-characterized signaling proteins such as ras and ras family members have been implicated in cell death pathways (Fernandez-Sarabia and Bischoff, 1993; Gulbins *et al.*, 1995; Wang *et al.*, 1995). However, the biochemical role of bcl-2 and its relatives remains unclear, the critical substrates of ICE family proteases are still unknown and, with the exception of apoptosis triggered by receptors which directly engage the apoptotic proteolytic machinery (Boldin *et al.*, 1996; Muzio *et al.*, 1996), the signaling events that regulate apoptosis remain to be determined. In order to address these issues, we have turned to a biochemically tractable *in vitro* model system for the analysis of apoptosis.

Recently, Newmeyer *et al.* reported the development of an apoptotic *Xenopus* egg extract (Newmeyer *et al.*, 1994). It is likely that the ability of these extracts to undergo apoptosis reflects the process of oocyte atresia *in vivo*, wherein oocytes which have matured but have not been laid are resorbed apoptotically (Tilly *et al.*, 1991; Hsueh *et al.*, 1994). The main difference between apoptotic egg extracts and the better known extracts used to reconstitute nuclear and cell cycle processes lies in the hormonal regimen employed to produce the eggs. To lay eggs suitable for apoptotic extracts, the frogs are allowed a much longer interval between the hormonal stimulus for oocyte maturation [pregnant mare serum gonadotropin (PMSG) injection] and induction of ovulation [human chorionic gonadotrophin (HCG) injection]: this presumably triggers the atretic program. After several hours of incubation at room temperature, these extracts fragment nuclei in a manner strongly reminiscent of apoptotic nuclear disassembly. Nuclear fragmentation is easily distinguishable from mitosis in that the nuclear envelope does not break down, condensed DNA is distributed into many small membrane-enclosed structures and gel electrophoresis reveals the characteristic 'laddering' of DNA indicative of internucleosomal cleavage. Nuclear fragmentation is inhibited completely by addition of bcl-2 protein, strongly suggesting that the similarity to *in vivo* apoptosis is more than superficial. It is interesting to note that apoptosis in these extracts requires a subcellular fraction containing mitochondria. Specifically, high speed centrifugation of the extract abolished the capacity for execution of apoptosis, which was restored upon re-addition of a mitochondria-enriched fraction of the extract (Newmeyer *et al.*, 1994). Recently Liu *et al.* (1996)

Fig. 1. Apoptosis in the egg extract. (A) Crude egg extract (unfractionated) was incubated at room temperature in the presence of sperm chromatin and an ATP-regenerating cocktail. Aliquots were withdrawn at 10 min intervals, fixed and visualized with the DNA stain, Hoechst 33258, and examined by fluorescence microscopy. Pictured is a representative intact nucleus 60 min after the start of the room temperature incubation. All nuclei in this extract exhibited a similar morphology, with intact nuclear envelopes, as confirmed by phase contrast microscopy, and decondensed DNA. (B) Reconstituted extract (membrane and cytoplasm) was incubated in the presence of an ATP-regenerating cocktail for 2.5 h. An execution extract to which sperm chromatin had been added was diluted 1:10 with the pre-incubated reconstituted extract and examined at 10 min intervals, as in (A). By 60 min, essentially all of the nuclei in the extract (2000/ μ l) had undergone apoptotic degeneration. (C) Extracts were treated as in (B), but Ac-YVAD-cmk peptide was added to the execution extract at a final concentration of 10 μ M.

reported that cytochrome c is required for apoptosis in HeLa cell extracts which can recapitulate apoptotic events *in vitro*. It remains to be determined if this is the mitochondrial component required to carry out apoptosis in egg extracts.

Correlative evidence from a number of different systems suggests that phosphotyrosine signaling pathways may play a role in the regulation and/or implementation of apoptotic programs. Tyrosine phosphorylation of cellular proteins is elevated (in some cases extremely rapidly) by a variety of apoptotic inducers, including staphylococcal enterotoxin B priming of splenic T cells, anti-CD3 monoclonal antibody treatment of thymic cells and taxol treatment of ovarian cells (Liu *et al.*, 1994; Migita *et al.*, 1994). However, the means by which tyrosine phosphorylation contributes to apoptotic signaling and the specific tyrosine kinases/phosphatases and substrates which are important in this process have not been defined.

Under many circumstances, signal transduction mediated by tyrosine phosphorylation involves interactions between tyrosine-phosphorylated proteins (such as growth factor or cytokine receptors) and proteins containing src homology 2 (SH2) domains (reviewed in Feller *et al.*, 1994b; Cohen *et al.*, 1995; Pawson, 1995). These protein domains, consisting of ~100 amino acids, mediate protein-protein interactions through avid sequence-specific binding of phosphotyrosine. While SH2 domains are found in a wide variety of signaling proteins, their function as mediators of protein-protein interactions is most evident in a class of molecules known as adaptor proteins where the polypeptide sequence consists largely of SH2 domains coupled to another modular protein domain (SH3), which binds to specific proline-rich sequences. Proteins which fall into this class, such as crk, nck and Grb2, are believed to act as adaptors by physically joining proteins required for the transmission of cellular signals (e.g. signals regulating cell proliferation).

Here, we have investigated the potential involvement of tyrosine kinase signaling pathways by testing the effects of a panel of phosphotyrosine-directed reagents in the *Xenopus* apoptotic extract. We have assayed the ability of various isolated recombinant SH2 domains to interfere with apoptosis in the extract, and show that the SH2

domain from the adaptor protein crk effectively and specifically blocked apoptosis. Moreover, apoptosis was inhibited upon crk immunodepletion from the extracts or following addition of anti-crck antisera to the extracts. Inhibition could be reversed by addition of recombinant *Xenopus* crk protein. We conclude that apoptosis *in vitro* involves phosphotyrosine signaling and requires the adaptor protein, crk.

Results

In order to facilitate further biochemical fractionation and analysis of the apoptotic egg extracts, we have modified the protocol of Newmeyer *et al.* (1994), exploiting their observation that a mitochondrial component is required for *in vitro* apoptotic nuclear fragmentation. Ultracentrifugation of crude interphase egg extracts at 250 000 g produces cytoplasmic and membrane fractions which are cleanly separated from mitochondria, ribosomes and other large organelles. A combined cytoplasmic and membrane fraction is fully competent to form nuclei *in vitro* (when reconstituted with an ATP-regenerating cocktail and sperm chromatin), but such 'reconstituted extracts' do not undergo apoptosis, even when prepared from eggs laid by frogs treated with the proper hormone regimen. Rather, we have found that nuclei formed in reconstituted extracts are completely stable over a 6 h time course. Conversely, nuclei formed in crude extracts (containing mitochondria) derived from the same eggs underwent apoptotic degeneration within 5 h. Although the reconstituted extract could not sustain full blown apoptosis, it did develop latent apoptotic activity upon incubation for 2.5 h at room temperature. Apoptotic activity was manifested upon transfer of one-tenth volume of the pre-incubated reconstituted extract into a crude extract containing freshly assembled nuclei. As visualized by fluorescence microscopy of samples stained with Hoechst 33258 dye, the nuclei underwent rapid degeneration (between 30 and 90 min, depending upon the individual extract) in a manner morphologically identical to that obtained by prolonged incubation of the crude extract (Figure 1B).

As reported by Newmeyer, *in vitro* nuclear fragmentation is inhibitable by bcl-2 (Newmeyer *et al.*, 1994). In

Table I. Generation of latent apoptotic activities

Time of pre-incubation of reconstituted extract (h)	Time of apoptotic nuclear fragmentation (min)
0	>240
0.5	>240
1.0	>240
1.5	>240
2.0	>240
2.5	60
3.0	60
3.5	60
4.0	60

addition, we found that DNA extracted from fragmenting nuclei exhibited laddering characteristic of apoptosis (data not shown), and that the nuclear fragmentation was inhibitable by addition of 10 μ M acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk), a peptide inhibitor of ICE-like proteases (Figure 1C). Thus, *in vitro* nuclear fragmentation faithfully reproduces the characteristics of apoptosis *in vivo*. The photomicrographs shown are highly representative in that apoptotic fragmentation was highly synchronous in a given extract, proceeding to completion within 10 min even at concentrations reaching thousands of nuclei per microliter of extract.

To determine the time required to generate latent apoptotic activity in reconstituted extracts, we pre-incubated the extracts for various times and then transferred aliquots (1/10 volume) into crude extracts containing newly formed synthetic sperm nuclei. A minimal pre-incubation of 2.5 h was required to promote accelerated apoptosis (scored within 2 h of transfer to the crude extract, Table I). Pre-incubations of >2.5 h did not alter the kinetics of subsequent apoptosis, suggesting that a threshold level of apoptotic inducers is achieved within 2.5 h, after which processes unique to the crude extract become rate-limiting for apoptosis. Although we have observed a good deal of extract-to-extract variability in the kinetics of apoptotic 'execution' in crude extracts, 2.5 h consistently is the minimal pre-incubation time for all reconstituted extracts we have tested.

For ease of description, the pre-incubated extract consisting of reconstituted membrane and cytoplasmic fractions will hereafter be referred to as the 'latent extract' (during which latent apoptotic activities are developed) and the crude extract containing nuclei will be referred to as the 'execution extract' (wherein apoptotic execution and nuclear fragmentation occur).

Membrane and cytoplasmic fractions are both required to generate apoptotic activities

Membrane and cytoplasmic fractions were pre-incubated ('aged') separately and tested by dilution into an execution extract as above. Neither fraction aged alone promoted accelerated apoptosis (Figure 2B and C). This might indicate that each aged fraction contains distinct essential apoptotic activities, or that interaction between the fractions during the aging incubation is essential for development of one or more activities. To distinguish between these options, we combined the separately aged membrane and cytoplasmic fractions in appropriate proportions before adding them to the execution extract. This did not result

in accelerated apoptosis (data not shown), suggesting that generation of apoptotic activity requires interaction between the two fractions.

In a separate experiment, a full latent extract (membrane plus cytoplasm) was aged to allow development of apoptotic activities, and then separated into membrane and cytoplasmic fractions. Under these conditions, the cytoplasmic fraction (but not the membrane fraction) was able to promote accelerated apoptosis upon addition to the execution extract (Figure 2D). The simplest interpretation of these results is that interaction between cytoplasmic and membrane fractions during the aging incubation generates an essential apoptotic activity which then remains exclusively in the cytoplasm. A more complex possibility is that the apoptotic activity generated by interaction between the fractions resides in both membrane and soluble fractions (and can be provided by the soluble fraction alone after incubation with the membrane), but that an additional essential activity resides exclusively in the cytoplasm (explaining the inability of the recovered aged membrane to accelerate apoptosis in the execution extract).

A final experiment supports this more complex explanation, and suggests that at least two apoptotic activities are generated during the aging incubation. In this experiment, two separate aging incubations were performed. In the first, a full latent extract was aged and the membrane fraction subsequently was isolated. As before, this fraction was unable to promote accelerated apoptosis despite the fact that this membrane had interacted with cytoplasm. In the second, cytoplasm was incubated in the absence of membrane. In the absence of interaction, this fraction was also unable to promote apoptosis. However, when these separately aged fractions were combined and then immediately diluted into the execution extract, apoptosis was greatly accelerated (Figure 2E). This suggests that a minimum of two apoptotic activities are generated during the aging incubation, both of which are essential. The first requires interaction between membrane and cytoplasm, and then resides in both fractions. The second activity develops in the cytoplasmic fraction alone.

Tyrosine kinase signaling pathways are essential for apoptosis *in vitro*

As discussed in the Introduction, several lines of evidence have implicated tyrosine kinase signaling pathways in apoptotic induction. Addition of 20 mM phosphotyrosine was reported to inhibit apoptosis in *Xenopus* egg extracts (Newmeyer *et al.*, 1994), a finding which we have confirmed. Furthermore, while apoptosis was severely delayed (for 2–3 h) upon phosphotyrosine addition to the latent extract, it was not delayed upon phosphotyrosine addition to the execution extract (Figure 3A), suggesting that important phosphotyrosine signaling events must occur during the latent phase.

Given that high concentrations of isolated phosphotyrosine can bind to SH2 domains, we considered the possibility that SH2-phosphotyrosine interactions might be critical for apoptotic induction during the latent phase preceding the appearance of morphological apoptosis. This hypothesis would predict that addition of free SH2 domains (of the appropriate specificity class) to the extract might compete with endogenous SH2-containing proteins for

Fig. 2. Membrane and cytoplasmic fractions are both required during the latent phase of apoptosis. In all cases, an ATP-regenerating cocktail was added during the incubations, and sperm chromatin (final concentration 2000/ μ l) was added to the execution extract. **(A)** A full latent extract was incubated for 2.5 h and diluted 1:10 into a crude execution extract. This led to synchronous apoptotic fragmentation of nuclei within 60–90 min, depending upon the extract. **(B)** The cytoplasm alone was incubated for 2.5 h and diluted into the execution extract. Apoptosis did not occur over 2–4 h, depending on the length of the particular experiment. **(C)** The membrane fraction alone [amount equivalent to that present in (A)] was incubated for 2.5 h at room temperature in buffer and then transferred 1:10 into execution extract. Apoptosis did not occur. **(D)** A full latent extract, as in (A), was incubated for 2.5 h. A portion of the extract was centrifuged at 200 000 g to obtain pure cytoplasm. This re-isolated cytoplasm was diluted into execution extract (1:10), promoting rapid apoptosis (30–90 min). The membrane fraction was re-isolated free of cytoplasmic contaminants by centrifugation through 0.5 M sucrose. Pure membrane was added to the execution extract at 1:100 (equivalent to a 1:10 dilution of the original full latent extract, since membrane is present in the full extract at one-tenth the volume of the cytoplasm). This did not promote apoptosis. **(E)** Membrane re-isolated as in (D) was mixed 1:10 with independently aged cytoplasm. This mixture was then diluted 1:10 into execution extract, thereby promoting apoptotic fragmentation of the resident nuclei.

Fig. 3. Phosphotyrosine inhibits apoptosis. (A) Phosphotyrosine (final concentration 20 mM) was added to the latent extract at the beginning of a 2.5 h room temperature incubation. After transfer of 1:10 volume of this latent extract into crude extract containing sperm nuclei, samples were incubated at room temperature and then stained with Hoechst dye and observed by fluorescence microscopy. Nuclei in this extract never entered apoptosis during the course of the experiment (3 h). (B) Phosphotyrosine (20 mM final concentration) was added to the crude execution extract at the time of transfer of 1/10 volume of the latent extract. Synchronous apoptotic fragmentation of nuclei in this extract was observed within 80 min. Samples were observed by fluorescence microscopy as in (A).

binding to critical phosphotyrosine-containing proteins, thereby inhibiting apoptosis in the extract. To determine whether this was the case, we produced a panel of seven SH2 domains with various specificities as GST-SH2 fusion proteins in bacteria, and added them individually to the latent extract for the entire 2.5 h pre-incubation period. GST-SH2 domains derived from the signaling proteins *abl*, *crk*, *ras* GAP, *Grb2*, *lck*, *nck* and phospholipase C γ (PLC γ) were added to a final concentration of ~35 ng/ μ l (0.1% of total extract protein). In the particular experiment shown in Figure 4, the control extract entered apoptosis in 70 min (with 100% nuclear fragmentation in 80 min), extract containing the *Grb2* SH2 domain delayed apoptosis by ~10 min and extract containing the *crk* SH2 never entered apoptosis (over a 2.5 h time course). Extracts containing even 4-fold more *abl*, *ras* GAP, *lck*, PLC γ or *nck* SH2 proteins entered apoptosis at the same time as control extracts. These findings were reproducible in several independent experiments, and strongly suggest that interruption of SH2-phosphotyrosine interactions in the extract can prevent the development of apoptotic activity during the latent phase. Furthermore, the surprising potency and specificity of inhibition displayed by the *crk* SH2 domain suggested that *crk* itself (or another protein with a similar specificity SH2 domain) might play a role in apoptotic signaling.

***Crk* is required for apoptosis**

To explore the potential role of *crk* in apoptosis further, we produced GST fusion proteins with wild-type chicken *crk*, *crk* mutated in the SH2 domain (R38 $\rightarrow$ K, unable to bind phosphotyrosine) and the *crk* N-terminal SH3 domain. These proteins were added individually to latent extracts at a concentration of ~35 ng/ μ l and, following the standard 2.5 h incubation, the extracts were tested for their ability to inhibit apoptosis upon dilution into the execution extract. Like the *crk* SH2 domain, addition of the *crk* SH3 domain or the *crk* R38K proteins completely inhibited apoptosis. In contrast, addition of wild-type *crk* had no effect (Table II). These results are precisely what would

Fig. 4. The SH2 domain of *crk* inhibits apoptosis. GST-SH2 domains derived from *abl* (A), *Grb2* (B), *crk* (C), *lck* (D), *ras* GAP (E), PLC γ (F) and *nck* (G) were each added (35 ng/ μ l final concentration) to the latent extract (membrane plus cytosol) at the beginning of a 2.5 h room temperature incubation. At the end of this incubation, a crude execution extract containing 2000 nuclei/ μ l and an ATP-regenerating system was diluted with 1:10 volume of the various latent extracts. Samples were withdrawn at 10 min intervals, stained with Hoechst 33258 and visualized by fluorescence microscopy.

be anticipated if *crk* itself is necessary to induce apoptosis: addition of extra wild-type *crk* should not impede the apoptotic signal, but either the R38K mutant or the isolated SH3 domain might compete with the endogenous *crk* SH3 for relevant SH3 binding proteins, acting as dominant-negative inhibitors of apoptosis.

We also tested GST fused to wild-type *Grb2* and *Grb2* mutated in the SH2 domain. Like the *Grb2* SH2 domain, the R86K mutant of *Grb2* produced a slight delay in apoptosis. Wild-type *Grb2* had no effect (data not shown). The potential significance of these results will be discussed further below.

If the mutant *crk* proteins are indeed inhibiting apoptosis

by binding to endogenous crk partners in the extract, then removal of these crk-interacting proteins from the extract should interrupt apoptotic signaling. To determine if this was the case, we linked the wild-type GST–crk to glutathione–Sepharose and added the resulting ‘crk beads’ to the latent extract for the 2.5 h incubation. Crk beads were then removed from the extracts, along with any bound proteins, by gentle centrifugation. As shown in Table II, extracts depleted with crk beads (but not with beads linked to GST protein alone), failed to promote apoptosis in the execution extract. Since addition of soluble wild-type crk fused to GST had no effect (above), the inhibition observed with the crk bead depletion protocol indicates that crk binding proteins in the extract are essential for apoptosis, and supports the idea that crk mutants or individual domains inhibit apoptosis through unproductive ‘dominant-negative’ interactions with such crk binding proteins.

Table II. Apoptosis and mutants of crk

	Ability to inhibit apoptosis upon addition to extract	Ability to inhibit apoptosis when used to deplete extract of binding proteins
Full-length crk	– ^a	+ ^b
crk SH2	+	+
crk SH3	+	+
crk R38K	+	+
GST	–	–

^aThe – indicates that apoptosis occurred between 50 and 90 min over a series of experiments.

^bThe + indicates that apoptosis was inhibited and did not occur over the course of any given experiment (140–240 min, depending on the experiment).

As expected, the GST–crk SH2 and GST–crk SH3 proteins, but not other GST–SH2 proteins, were also effective in the bead depletion experiment (Table II). These results support a role for crk binding proteins in apoptotic signaling, and also suggest that both SH2- and SH3-mediated interactions are important.

These experiments are strongly suggestive of a role for the endogenous *Xenopus* crk and its binding partners in promoting apoptosis. To confirm this, we sought to eliminate endogenous crk directly from the extract. Immunoblotting of total *Xenopus* extract with antisera against chicken crk identified a band of the predicted molecular weight for crk (35 kDa; Figure 5A). Immunoprecipitation of crk protein from 30 µl of latent extract with increasing quantities (0, 1, 5, 10 µl) of anti-chicken crk showed that even at levels as low as 5 µl of antisera, the majority of the endogenous crk was depleted from the extract (Figure 5A). Latent extracts were immunodepleted with either crk antisera or pre-immune sera and assayed for their ability to accelerate apoptosis in an execution extract. Extracts depleted with anti-crck antisera were no longer able to trigger apoptosis (Figure 5C, left panel), while depletion with pre-immune sera had no effect (Figure 5C, middle). As a specificity control, we obtained antisera to the related adaptor protein, nck. This antiserum was also able to immunodeplete a protein of the expected molecular weight (Figure 5B). Unlike crk depletion, however, nck depletion had no effect (Figure 5C, right panel). We also attempted similar experiments with Grb2 depletion, but we were unable to immunodeplete *Xenopus* Grb2 efficiently using several different antisera.

In an alternative approach to blocking endogenous crk function in the extract, we added either pre-immune or

Fig. 5. Depletion of crk inhibits apoptosis. (A) Crk was immunoprecipitated from 30 µl of the cytosolic fraction of the latent extract using increasing amounts of antisera linked to protein A–Sepharose. The supernatants remaining after removal of the antibody–antigen complexes (by pelleting of the Sepharose beads) were processed for Western blotting with anti-crck antisera. Lane 1, undepleted extract; lane 2, 0 µl; lane 3, 1 µl; lane 4, 5 µl; and lane 5, 10 µl of anti-crck. In lane 6, pre-immune serum was used for the initial immunoprecipitation. (B) Lane 1: 10 µl of egg cytosol from a latent apoptotic extract was resolved by SDS–PAGE and processed for Western blotting with anti-nck antisera. Lane 2: cytosol was immunodepleted with anti-nck antisera (as done for anti-crck in A) and the depleted cytosol was processed for Western blotting with anti-nck sera. (C) Cytosol depleted with anti-crck antisera (left panel), pre-immune sera (middle panel) or anti-nck sera (right panel) was incubated in the presence of membrane and an ATP-regenerating system at room temperature for 2.5 h. Three µl of each of these was added to individual 27 µl aliquots of crude execution extract containing sperm chromatin and an ATP-regenerating system. Samples were observed following Hoechst staining by fluorescent microscopy. Photomicrographs were taken 60 min after transfer of the latent extract mixture into the crude extract, but the crk-depleted sample never entered apoptosis throughout the course of the experiment (>2.5 h).

Fig. 6. Addition of crk antisera to the latent extract inhibits apoptosis. (A) Three μ l of crk antisera was added to 30 μ l of latent extract (membrane plus cytosol) prior to the standard 2.5 h room temperature incubation period. Then 3 μ l of this mixture was added to 27 μ l of crude execution extract and observed as described in Figure 5. (B) Samples were processed as in (A), but 3 μ l of pre-immune sera, rather than crk antisera, was used. (C) Samples were processed as in (A), but recombinant *Xenopus* GST-cr_k was co-added with the crk antisera.

anti-cr_k antisera directly to the extract. We chose to add 3 μ l of antisera to 30 μ l of latent extract because, as shown above, this volume of antisera could immunodeplete most of the relevant protein from the extract. Moreover, we did not wish to risk compromising the function of the latent extract simply by virtue of dilution with antisera. Addition of anti-cr_k sera, but not pre-immune or anti-nck sera (data not shown), completely blocked apoptosis (Figure 6A and B), supporting the conclusion that cr_k protein is required for *in vitro* apoptosis.

To demonstrate that the inhibitory effect of cr_k antibody addition was specific, we added recombinant cr_k protein to the extract along with the cr_k antisera. By screening a *Xenopus* cDNA library with a chicken cr_k probe, we cloned a *Xenopus* cr_k homolog and used it to produce recombinant GST-cr_k fusion protein for addition to the extracts. Co-addition of *Xenopus* cr_k, which is 90% homologous to chicken c-cr_k II (the more abundant of the two known isoforms of c-cr_k) at the amino acid level (Figure 7) (Matsuda *et al.*, 1992; Reichman *et al.*, 1992), reversed the inhibitory action of the cr_k antisera, restoring the ability of the extract to promote apoptosis (Figure 6C). Thus, the inhibitory action of the anti-cr_k antiserum is exerted through the same epitopes that are present on endogenous cr_k.

In the course of performing these experiments, we produced several extracts which were unable to promote apoptosis (probably due to an insufficient interval between PMSG priming and HCG injection during egg production). On several occasions, addition of cr_k protein conferred the ability to induce apoptosis on such 'insufficiently apoptotic' latent extracts (E.K.Evans and S.Kornbluth, unpublished observations). This observation is consistent with the hypothesis that cr_k is a positive regulator of apoptosis, and may indicate that a step mediated by cr_k is a rate-limiting component in some extracts.

Discussion

Apoptosis *in vitro*

The unmatched ability of *Xenopus* egg extracts to reconstitute complex cellular functions in a cell-free system has provided many important insights into various processes, including nuclear protein import and cell cycle control. The results described here confirm and expand the utility of appropriate *Xenopus* egg extracts for studying apoptosis, and demonstrate their value for isolating and analyzing

novel components of apoptotic signaling pathways. In a number of different mammalian systems, oocyte atresia has been demonstrated to occur via apoptotic pathways. In mammals (as in frogs), hormonal perturbations can initiate this process (Hsueh *et al.*, 1994). We suggest that the frog egg extract may provide an excellent *in vitro* model for the apoptotic process in general and for the *in vivo* process of oocyte atresia, in particular.

The authenticity of the extract as a model for *in vivo* apoptosis was strongly suggested by the fact that the *in vitro* apoptotic process could be inhibited by bcl-2 protein, and is confirmed by our finding that Ac-YVAD-cmk (an inhibitor of ICE proteases) can also inhibit apoptosis effectively in this system.

We have refined the original system described by Newmeyer *et al.*, separating the assay into a latent step and an execution step, which are carried out in separate extracts. As reported by Newmeyer *et al.*, rapidly sedimenting components (most likely associated with mitochondria) are required for apoptosis in the execution extract (Newmeyer *et al.*, 1994). The lack of a requirement for mitochondria in the latent extract greatly simplifies its manipulation and allows the frozen storage of this extract. Using this system, we have shown that membrane and soluble components are both required for the development of apoptotic potential during the latent phase. While a subset of necessary apoptotic activities develop in a membrane-free soluble extract, others require the interaction of membrane and soluble factors. These findings reveal an unexpected complexity in the signaling processes that occur during the latent phase.

Since apoptotic extracts are only produced if the frog laying the eggs has been treated with a particular hormone regimen, we would expect that signals which initiate the apoptotic program have occurred *in vivo* before the extract is made. None the less, in order for the latent extract to trigger apoptosis upon transfer to the execution extract, a 2.5 h pre-incubation was required. The nature of the biochemical reactions responsible for generating apoptotic activities are, at this time, unknown. However, signal transduction events do not, in general, tend to require a time interval as long as 2.5 h. We propose that pro- and anti-apoptotic pathways are both operative in the latent extracts and that it is the balance between the two which determines whether the extract will become apoptotic. A similar scenario *in vivo* may explain the observed vari-

Crk2x	1	.MAGNFDSEDRASWYWGKQNRQEAVALLQQRHGVFLVRDSTIPGDYVLSVSENSKVSH
Crk2h	1	.MAGNFDSEDRSSWYWGRLSRQEAVALLQQRHGVFLVRDSSSTSPGDYVLSVSENSRVSH
Crk2c	1	.MAGQFDSEDRGSWYWGRLSRGDAVALLQQRHGVFLVRDSCSTIPGDYVLSVSESSRVSH
Crk1h	1	MSARFDSSDRSAWYMGFVSRQEAQRLQQRHGMFLVRDSSSTCPGDYVLSVSENSRVSH
Crk2x	60	YIINSVT.....NNRQSSSTAGMVQSRFRIGDQEFDSLPTLLEFYKIHVLDTTTTLIEPV
Crk2h	60	YIINSSSGRPPVPVPPSPAQPPPGVSPSRFRIGDQEFDSLPPALLEFYKIHVLDTTTTLIEPV
Crk2c	60	YIIVNSGGPAGGRRAGGEGPGAPGLNPTFRFRIGDQEFDSLPTLLEFYKIHVLDTTTTLIEPV
Crk1h	61	YIINS.....LPNRRFKIGDQEFDSLPPALLEFYKIHVLDTTTTLIEPA
Crk2x	113	SK.....SKQ.SGVIQRQEEVEYRALFDFLGNDDEDLPFKKGDILRIRKPEEQWWN
Crk2h	119	SR.....SRQSGVILRQEEAEYVRALFDFNGNDEEDLPFKKGDILRIRKPEEQWWN
Crk2c	120	SR.....SRQNSGVILRQEEVEYVRALFDFNGNDEEDLPFKKGDILKIRKPEEQWWN
Crk1h	103	PRYPSPPMGSVSAPNLPTAEDNLEYVRTLDFPGNDAEDLPFKKGELVITIKPEEQWWS
Crk2x	165	AEDSDGKRGMPVPYVEKYRPPSSPGSALIGGNQENSHPOPLGGPEPG.PYAQPSVNTPL
Crk2h	172	AEDSCKRGMPVPYVEKYRPPASASVSALIGGNQEGSHPOPLGGPEPG.PYAQPSVNTPL
Crk2c	173	AEDMDGKRGMPVPYVEKCRPSSASVSTLTGGNQDSSHPQPLGGPEPG.PYAQPSINTPL
Crk1h	163	ARNKDKGRVGMIPVPYVEKLVRSPPH.....GKHGNRNSNSYCTPEPAHAYAQPQTTPPL
Crk2x	224	PNL.....QNGPIARVIQKRVPNAYDKTALALEVGLVKVTKINVSQOWE
Crk2h	231	PNL.....QNGPIARVIQKRVPNAYDKTALALEVGLVKVTKINVSQOWE
Crk2c	232	PNL.....QNGPIARVIQKRVPNAYDKTALALEVGLVKVTKINVSQOWE
Crk1h	217	PAVSGSPGAAITPLPSTQNGPVFAKAIQKRVPCAYDKTALALEVGLVKVTKINVSQOWE
Crk2x	270	GECNGKRYGHFPFTHVRLLE.QNPDEDFS
Crk2h	277	GGCNGKRGHFPFTHVRLLDQONPDEDFS
Crk2c	278	GECNGKRGHFPFTHVRLLDQONPDEDFS
Crk1h	277	GEVNGKRGHFPFTHVRLLDQONPDENE.

Fig. 7. *Xenopus* crk is highly homologous to chicken crk2. Comparison of amino acid sequences of the cloned *Xenopus* crk homolog to the chicken crk2, human crk2 and human crkL sequences. The degree of identity (shaded black) and similarity (shaded gray) for the pairwise combinations are: *Xenopus* crk to human crk2, 84 and 91%; *Xenopus* crk to chicken crk2, 81 and 90%, *Xenopus* crk to human crkL, 66 and 79%. Sequences were aligned using the Pileup program of the GCG package, and the alignment was printed using the program Boxshade. Using chicken crk as a probe, the *Xenopus* crk was isolated from a λ -Zap cDNA library constructed from stage 11.5 *Xenopus* embryos. Crk2x = *Xenopus* crk2; Crk2h = human crk2; Crk1h = human crkL.

ability in the duration of the latent phase in cells condemned to die.

Direct demonstration of the activation of apoptotic proteases in *Xenopus* egg extracts has been shown recently by Cosulich *et al.* (1996). Our observation that the Ac-YVAD-cmk peptide inhibited apoptosis upon incubation in the execution extract, but not when added solely to the latent extract, supports the notion that ICE proteases are required at a late stage of apoptosis, perhaps coinciding with the onset of visible cell death. This would place the proposed 'protease cascade' involving ICE-like proteases downstream of the signaling events in the latent extract (reviewed in Chinnaiyan and Dixit, 1996).

Phosphotyrosine signaling and apoptosis

As described in the Introduction, tyrosine phosphorylation of cellular proteins is elevated after treatment with a number of different inducers of apoptosis, suggesting that increasing tyrosine phosphorylation promotes apoptosis. However, apoptosis is inhibited when the total cellular phosphotyrosine is increased through overexpression of the tyrosine kinase v-abl or by treatment of cells with the phosphotyrosine phosphatase inhibitors PAO and vanadate (Chapman *et al.*, 1994; Yousefi *et al.*, 1994; McGahon *et al.*, 1995; Owen-Lynch *et al.*, 1995; Schieven *et al.*, 1995). Moreover, treatment of cells with tyrosine kinase inhibitors such as herbimycin or genistein has been reported to either block or promote apoptosis, depending

upon the particular apoptotic stimulus or cell type used. Similarly, specific phosphotyrosine phosphatases (FAP, HCP phosphatase) have been implicated in either stimulatory or inhibitory apoptotic pathways (Sato *et al.*, 1995; Su *et al.*, 1995). Thus, whether protein tyrosine phosphorylation favors or antagonizes apoptosis apparently depends upon the cell type being examined and upon the nature of the apoptotic trigger.

We have observed an increase in the number of tyrosine-phosphorylated proteins in the extract (by anti-phosphotyrosine immunoblotting) during the incubation of the latent extract, followed by a rapid dephosphorylation of tyrosine-phosphorylated proteins immediately upon transfer into the execution extract (E.K. Evans and S. Kornbluth, unpublished observations). This indicates that both increases and decreases in tyrosine phosphorylation of specific proteins at different stages may be required for implementation of the entire apoptotic program. This would suggest that the heterogeneous results obtained in different cell systems might be accommodated easily in a model whereby signaling events during the latent phase require increased tyrosine phosphorylation, but later events during the execution phase require a decrease in tyrosine phosphorylation. Confirmation of this model will require the identification of the key tyrosine-phosphorylated proteins. The results reported here constitute a first step in this direction, and implicate interactions between the SH2 domain of the adaptor protein, crk, and tyrosine-

phosphorylated proteins in the extract in the transmission of the apoptotic signal.

Crk and apoptosis

We have provided several lines of evidence supporting a positive role for cellular crk protein in apoptotic signaling pathways. (i) The crk SH2 domain inhibited apoptotic signaling. The specificity of this result is highlighted by the fact that the crk SH2 domain was the only one of the panel of SH2s examined which could prevent apoptosis effectively, even when present at one-quarter the concentration of the other SH2s. (ii) The crk SH3 domain also inhibited apoptotic signaling. Since the SH2 and SH3 domains are expected to interact with distinct sets of proteins, this suggests that at least two different crk binding proteins are essential for apoptosis. (iii) Depletion of crk-interacting proteins from the latent extract with crk beads (containing full-length crk) inhibited apoptotic signaling. (iv) Immunodepletion of endogenous crk inhibited apoptotic signaling. Immunodepletion of the adaptor protein, nck, did not inhibit apoptosis, providing a further specificity control. (v) Addition of anti-crak antibody inhibited apoptotic signaling. Furthermore, addition of recombinant *Xenopus* crk restored apoptosis to the blocked extract, demonstrating the crk specificity of antibody action. Again, addition of nck antibody had no effect. Together, these observations make a very strong case for the involvement of crk in apoptotic signaling. It is likely that all of the signaling events involving crk occur in the latent extract: addition of the crk SH2 domain had no effect on apoptosis when added only to the execution extract (data not shown), and depletion of crk only from the latent extract prevented apoptosis, despite reasonable levels of crk remaining in the execution extract.

While the specific function of the cellular adaptor protein, crk, has been elusive, the fact that an oncogenic variant of c-crak transforms cells in culture and induces tumors in animals implies a role for crk in the regulation of cell proliferation. Cellular transformation by the retrovirally encoded transforming derivative of c-crak (v-crak) is accompanied by an elevation in cellular phosphotyrosine, and the crk structure consists mainly of modular protein domains implicated in signal transduction (SH2 and SH3 domains) (Mayer *et al.*, 1988; Mayer and Hanafusa, 1990). This has led to the generally accepted notion that crk participates in mitogenic signal transduction pathways involving tyrosine phosphorylation. Our results suggest that crk also plays a positive role in apoptotic signaling, and it may, therefore, be worthwhile to determine whether v-crak transformation involves perturbing cell death pathways in addition to mitogenic pathways.

A number of crk binding proteins have been isolated from chicken and mammalian cells, including the adaptor protein SHC, the tyrosine kinase abl, the focal adhesion protein paxillin and two different GDP/GTP exchange factors for ras-like GTPases, Sos and C3G (Matsuda *et al.*, 1992; Birge *et al.*, 1993; Feller *et al.*, 1994a; Tanaka *et al.*, 1994; Schaller and Parsons, 1995). The possibility that crk-induced activation of C3G or Sos might be involved in apoptotic signaling is particularly attractive, given that several lines of evidence have implicated ras and ras family members in the induction of apoptosis. The possible

roles of these crk binding proteins in apoptotic signaling in the extract can now be addressed.

It is interesting to note that two different guanine nucleotide exchange factors, Sos and C3G, can interact with the SH3 domains of both crk and Grb2, although C3G exhibits greater specificity for crk (Knudsen *et al.*, 1994; Matsuda *et al.*, 1994; Tanaka *et al.*, 1994). Although problems with Grb2 antisera do not allow a definitive statement regarding the possible role of Grb2 in *in vitro* apoptosis, it is interesting that the K86 mutant of Grb2 and the isolated Grb2 SH2 domain delayed apoptosis, if only slightly. It is possible that the Grb2 SH3 domain of the K86 mutant inhibited functioning of endogenous crk protein in *trans* by sequestration of Sos, C3G or other factors which are also required for crk signaling. This would not explain the similar delay in apoptosis produced by the isolated Grb2 SH2 domain, but it would not be entirely unexpected for the isolated Grb2 SH2 to bind proteins in the extract normally bound by the crk SH2, because the isolated Grb2 SH2 exhibits a weak sequence selectivity (less than a quarter of that of the crk SH2) (Songyang *et al.*, 1993). Thus, the relatively weak effects obtained with Grb2 domains may reflect inefficient interference with crk function, rather than a direct role for Grb2 in apoptosis.

Inhibition of apoptosis by bcr/abl

A number of reports have suggested that cellular transformation by the oncoprotein bcr/abl is mediated, in part, through its ability to inhibit apoptosis. Specifically, expression of bcr/abl in several different cell types has been shown to confer resistance to induction of apoptosis by either chemotherapeutic agents or serum withdrawal (Chapman *et al.*, 1994; Yousefi *et al.*, 1994; Cortez *et al.*, 1995; Owen-Lynch *et al.*, 1995). These observations are particularly interesting in light of our data implicating crk in the transmission of apoptotic signals, since crk binding has been shown to alter the spectrum of proteins phosphorylated by Abl *in vitro*, and dominant-negative Crk mutants affect the ability of Abl, but not the epidermal growth factor (EGF) receptor, to activate MAP kinase pathways in transfected cells (Tanaka *et al.*, 1996). Moreover, c-abl protein has been shown to bind to and phosphorylate c-crak (Feller *et al.*, 1994a). It has been proposed that the SH2 domain of crk can interact intramolecularly with phosphotyrosine at the site of abl-induced phosphorylation, folding the crk molecule in such a way that it is impossible for crk SH3 domains to interact with their appropriate targets (Feller *et al.*, 1994a,b). Indeed the ability of crk SH2 to bind phosphotyrosine intramolecularly has been demonstrated recently using NMR spectroscopic analysis (Rosen *et al.*, 1995). Interestingly, addition of baculovirus-produced bcr/abl protein to the latent phase *Xenopus* egg (but not the execution extract) can inhibit apoptosis (E.K.Evans and S.Kornbluth, unpublished observations). It is tempting to speculate that the ability of bcr/abl to prevent apoptosis is mediated, at least in part, through the inhibitory phosphorylation of crk. In this regard, it should be noted that crkL, a protein very closely related to crk, is one of the major tyrosine-phosphorylated proteins in chronic myelogenous leukemia cells expressing bcr/abl (Nichols *et al.*, 1994). It will be interesting to determine whether replacement of the

endogenous crk protein with protein complexes containing a non-phosphorylatable mutant of crk (the site of abl phosphorylation has been mapped to Tyr221 of crk) will impair the ability of abl to inhibit apoptosis.

An alternative explanation for the ability of bcr/abl to inhibit apoptosis lies in the fact that abl can phosphorylate several of the known crk SH2-binding proteins (e.g. paxillin, SH2 and cbl) (Owen-Lynch *et al.*, 1995; Ribon *et al.*, 1995; Salgia *et al.*, 1995). It is possible that hyperphosphorylation of these binding proteins might titrate crk away from interactors required for the transmission of pro-apoptotic signals. This hypothesis may also be relevant to the observation that overexpression of activated alleles of the focal adhesion kinase (FAK) can prevent apoptosis of cells which have lost cell-cell contacts (Frisch *et al.*, 1996). Like abl, FAK can heavily phosphorylate a number of known crk binding proteins (p130^{cas}, paxillin) (Schaller and Parsons, 1995; Vuori *et al.*, 1996). Although speculative, these suggestions provide testable hypotheses for future examination.

The data presented here, implicating crk in apoptosis and demonstrating that both membrane and cytoplasmic components are required for apoptotic induction, pave the way for the identification and characterization of novel regulators of cell death. We anticipate that further fractionation of the apoptotic extracts will reveal a host of proteins required for apoptotic signaling. Moreover, the data presented here strongly suggest that at least a subset of them will be binding partners of the adaptor protein, crk.

Materials and methods

Preparation of apoptotic extracts

For induction of egg laying, mature female frogs were injected with 100 U of PMSG (Calbiochem) to induce oocyte maturation, followed (8–28 days later) by injection with HCG (USB). At 14–20 h after injection with HCG, eggs were harvested for extract production according to the following protocol. Jelly coats were removed from eggs by incubation with 2% cysteine (pH 8.0), washed three times in modified Ringers solution (MMR) (1 M NaCl, 20 mM KCl, 10 mM Mg SO₄, 25 mM CaCl₂, 5 mM HEPES pH 7.8, 0.8 mM EDTA), and then washed in egg lysis buffer [ELB; 250 mM sucrose, 2.5 mM MgCl₂, 1.0 mM dithiothreitol (DTT), 50 mM KCl, 10 mM HEPES pH 7.7]. Following addition of aprotinin and leupeptin (final concentration 5 µg/ml) and cytochalasin B (final concentration 5 µg/ml), eggs were packed by low speed centrifugation at 400 g and lysed by centrifugation at 10 000 g for 15 min. This crude or unfractionated extract constitutes the execution extract. To fractionate the extract into cytoplasmic and membranous components, it was centrifuged further at 55 000 r.p.m. (200 000 g) in a Beckman TLS-55 rotor for the TL-100 centrifuge. The cytosolic fraction was removed, re-centrifuged at 55 000 r.p.m. for an additional 25 min, aliquoted, frozen in liquid nitrogen and stored at –80°C. The membrane fraction was diluted into 1.5 ml of ELB and pelleted through a 5 M sucrose cushion for 20 min at 20 000 r.p.m. The isolated membrane was then aliquoted and stored at –80°C.

For apoptosis assays, the frozen membrane and cytosolic fractions were recombined by diluting the membrane 1:10 into the cytoplasm. An ATP-regenerating system was added (final concentration 2 mM ATP, 0.05 mg/ml creatine kinase and 20 mM phosphocreatine) and this reconstituted extract (latent extract) was incubated at room temperature for 2.5 h. After 2.5 h, latent extract was diluted into fresh execution extract 1:10. Sperm chromatin diluted to form 2000 nuclei/µl and an additional ATP-regenerating system (150 mM creatine kinase, 20 mM ATP, 2 mM EGTA and 20 mM MgCl₂) were added to the execution extract. Samples were withdrawn at 10 min intervals during incubation at room temperature and visualized for morphological changes characteristic of apoptosis by fluorescence microscopy following staining with Hoechst 33258 and formaldehyde fixation.

In some experiments, phosphotyrosine (20 mM, pH 7.0), GST fusion

proteins or antibodies were added at the start of the 2.5 h room temperature incubation. ICE protease inhibitor peptide, Ac-YVAD-cmk, was added (10 µM) at time of transfer into the execution extract.

Preparation of GST fusion proteins

Clones encoding all of the GST fusion proteins used in this study except the nck SH2 domain were generous gifts of Dr A.M.Pendergast. The GST–nck SH2 domain construct was produced by PCR amplification of human nck DNA with primers 5′/CCC CCG CCG CCG ATC ATG ATA AAT GCT TGA CAA 3′ and 5′/CCC CCG GAT CCT CAC TCA CTG GAA AGT TTG CTG GCA ATC 3′. Following amplification, the PCR products were digested with *NorI* and *BamHI* and cloned into the expression vector Gex 2N (a derivative of pGEX 2T, Pharmacia, containing a 3′ *NorI* cloning site).

The bacterial strain Topp1 (Stratagene) was transformed with each of the GST–protein constructs. Bacteria expressing the recombinant proteins were lysed on ice with 10 mg of lysozyme (Sigma) in 50 mM Tris pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF). Bacterial DNA was digested upon addition of 0.1% sodium deoxycholate, 15 mM MgCl₂ and 0.5 mg DNase I (Worthington Biochemical Corp.). Lysates were clarified by centrifugation at 11 000 r.p.m. for 45 min in a Sorvall SS34 rotor, and the supernatant was combined with 2 ml of fresh glutathione–Sepharose beads (Pharmacia). The glutathione–Sepharose beads were rotated at 4°C for 45 min, spun in a clinical centrifuge and the supernatant was discarded. The beads were then washed four times with 10 mM HEPES pH 8.0, 1 mM DTT and 150 mM NaCl. Full-length crk and grb2 proteins were prepared similarly.

For depletion experiments, beads coupled to GST proteins were stored on ice at 4°C with 5 µg/ml of aprotinin and leupeptin and used within 4 days. For addition experiments, the GST–proteins were eluted from glutathione–Sepharose beads with 5 mM glutathione (Sigma) in 10 mM HEPES, 1 mM DTT, pH 8.0. The eluate containing the GST–proteins was then concentrated by centrifugation in Centricon 30s (Amicon), exchanged into XB buffer (100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM K–HEPES pH 7.7 and 50 mM sucrose) and re-concentrated to 1–5 mg/ml. These proteins were aliquoted and stored at –80°C.

GST–protein bead depletion assays

GST–proteins coupled to glutathione–Sepharose beads produced by the method described above were washed three times with ELB. The GST–protein beads were then blocked by incubation with the cytoplasmic fraction of a non-apoptotic egg extract (i.e. an extract which had not been aged for 2.5 h at room temperature) for 30 min at 4°C. The bead–protein complex was then pelleted and washed twice more with ELB. Cytoplasmic latent apoptotic extract with an ATP-regenerating system was then added at 10 times the volume of beads and rotated at room temperature for 2.5 h. After pelleting the bead complex, the supernatant was recombined with membrane which had been aged for 2.5 h in separate cytosol and was ultracentrifuged through a 0.5 M sucrose cushion at 20 000 r.p.m. to remove any contaminating cytoplasmic components. The depleted cytosol recombined with ‘aged’ membrane was then transferred immediately 1:10 into execution extract and assayed for apoptosis as described above.

Antibody addition and immunodepletion assays

Antibodies raised in rabbit against full-length recombinant chicken crk and human nck proteins were used specifically to deplete the cytoplasmic fraction of the latent extract of either crk or nck. To determine the amount of antibody needed to immunodeplete crk or nck, protein A–Sepharose beads (Sigma) were first washed twice with ELB. After washing, the beads were pre-incubated with 10 mg/ml bovine serum albumin (Sigma) in ELB for 30 min at 4°C. The beads were washed twice more with ELB and 5 µl of Sepharose beads were incubated with either 0, 1, 5 or 10 µl of the appropriate antiserum at 4°C for 45 min. After two more washes with ELB, 30 µl of cytoplasm were added to each Sepharose–antibody complex and incubated at 4°C for 1 h. To determine the amount of crk or nck protein remaining after depletion with each antibody quantity, the beads were pelleted and the supernatants were processed for Western blotting. The samples were resolved by SDS–PAGE, transferred to PVDF membrane (Millipore) and probed with the appropriate antiserum. Blots were developed with horseradish peroxidase-linked goat anti-rabbit antibody and the renaissance chemiluminescent reagent from Dupont NEN.

Immunodepletion assays

For immunodepletion assays, protein A–Sepharose beads were washed, coupled to antibody and incubated with cytoplasm as described above.

After 1 h at 4°C, the antibody–PAS complex was pelleted. The supernatant was transferred to a fresh microfuge tube, recombined (10:1) with membrane from frozen stock and supplemented with an ATP-regenerating system. This depleted latent extract was incubated for 2.5 h at room temperature, diluted 1:10 into the execution extract and assayed for the ability to induce apoptotic nuclear fragmentation.

Antibody addition

As ~3 µl of antibody depleted crk from 30 µl of cytoplasm (Figure 6), a 1:10 dilution of crk antibody was added directly to the latent extract and incubated for 2.5 h at room temperature. Equal amounts of anti-nck antisera or pre-immune sera were added as controls. To rescue apoptosis in crk-depleted extracts, recombinant GST–chicken or *Xenopus* crk (~35 ng/µl) was added to the latent extract along with the anti-crck sera. This extract was incubated at room temperature for 2.5 h and then transferred to an execution extract.

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