

# Wee1-regulated Apoptosis Mediated by the Crk Adaptor Protein in *Xenopus* Egg Extracts

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**Abstract.** Many of the biochemical reactions of apoptotic cell death, including mitochondrial cytochrome c release and caspase activation, can be reconstituted in cell-free extracts derived from *Xenopus* eggs. In addition, because caspase activation does not occur until the egg extract has been incubated for several hours on the bench, upstream signaling processes occurring before full apoptosis are rendered accessible to biochemical manipulation. We reported previously that the adaptor protein Crk is required for apoptotic signaling in egg extracts (Evans, E.K., W. Lu, S.L. Strum, B.J. Mayer, and S. Kornbluth. 1997. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:230–241). Moreover, we demonstrated that removal of Crk Src homology (SH)2 or SH3 interactors from the extracts prevented apoptosis. We now report the finding that the relevant Crk SH2-interacting pro-

tein, important for apoptotic signaling in the extract, is the well-known cell cycle regulator, Wee1. We have demonstrated a specific interaction between tyrosine-phosphorylated Wee1 and the Crk SH2 domain and have shown that recombinant Wee1 can restore apoptosis to an extract depleted of SH2 interactors. Moreover, exogenous Wee1 accelerated apoptosis in egg extracts, and this acceleration was largely dependent on the presence of endogenous Crk protein. As other Cdk inhibitors, such as roscovitine and Myt1, did not act like Wee1 to accelerate apoptosis, we propose that Wee1–Crk complexes signal in a novel apoptotic pathway, which may be unrelated to Wee1's role as a cell cycle regulator.

**Key words:** apoptosis • caspase • Crk • Wee1 • *Xenopus*

## Introduction

The removal of harmful or unnecessary cells through apoptosis, or programmed cell death, is crucial to the health of multicellular organisms (for reviews see Vaux and Korsmeyer, 1999; Green, 2000). Apoptosis is important in a variety of physiological contexts, including maintenance of the immune system, embryological development, and organismal homeostasis. Unlike necrosis, apoptotic cell death is compatible with these biological processes as it allows removal of individual cells without an accompanying inflammatory response. Dying cells are quickly destroyed, packaged into membrane-bound vesicles, and phagocytosed by neighboring cells.

Although apoptotic signaling pathways differ between cell types, in most cells the key executioners of the apoptotic process are members of a protease family known as the caspases (Alnemri, 1997). These proteases, which

share homology with the *Caenorhabditis elegans* death gene product, CED-3, are synthesized in a zymogenic form and activated either by proximity-induced autoprocessing or cleavage in trans by other caspases. Once activated, caspases undermine cellular integrity by cleaving key cellular substrates such as nuclear lamins and gelsolin (for review see Thornberry and Lazebnik, 1998).

In many apoptotic cells, caspase activation is preceded by release of cytochrome c from the intermembrane space of the mitochondria to the cytoplasm (for review see Green and Reed, 1998). Once released into the cytosol, cytochrome c serves as an activating cofactor in a multimeric structure known as the “apoptosome,” comprised of a caspase (caspase-9), an ATP-binding protein (Apaf-1), and ATP (or dATP) (Liu et al., 1996; Li et al., 1997; Zou et al., 1999). The apoptosome, once activated, induces proteolytic activation of procaspase-9, which subsequently cleaves and activates a workhorse of execution, caspase-3. Hence, the mitochondrial release of cytochrome c is a paramount site of regulation of programmed cell death, in

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particular, by the Bcl-2 family of proteins (for review see Gross et al., 1999). Proapoptotic members of the family, such as Bax, promote the release of cytochrome c, while antiapoptotic members, such as Bcl-xL, inhibit translocation of cytochrome c from the mitochondrial intermembrane space to the cytosol.

Apoptosis has been very well conserved in metazoans. Not only are homologous apoptotic signaling molecules conserved among flies, frogs, worms, and mammals, but apoptotic regulators in one system can often functionally substitute for those in another (e.g., Hengartner and Horvitz, 1994; Newmeyer et al., 1994; Evans et al., 1997a; Kuwana et al., 1998; Dorstyn et al., 1999). In keeping with this evolutionary conservation of the apoptotic program, the dramatic biochemical and morphological events of apoptosis can be recapitulated in a cell-free system derived from eggs of the frog, *Xenopus laevis* (Newmeyer et al., 1994; Evans et al., 1997b; Kluck et al., 1997b). When these extracts are incubated at room temperature, hallmark apoptotic activities, such as mitochondrial cytochrome c release, caspase activation, cleavage of apoptotic substrates, and DNase activation can be observed. Furthermore, nuclei added to these extracts undergo morphological changes characteristic of apoptosis, including chromatin condensation, membrane vesiculation, and ultimately, complete nuclear fragmentation. Although the physiological basis for this apoptotic program has not been precisely defined, it has been speculated that these extracts serve as an in vitro model for oocyte atresia, wherein matured oocytes that are not laid as eggs are reabsorbed by apoptotic cell death (Hughes and Gorospe, 1991; Smith et al., 1991; Tilly et al., 1992; Newmeyer et al., 1994).

Characterization of *Xenopus* egg extracts by several laboratories has established that the egg extract displays appropriate biochemical responses to common inhibitors of apoptosis such as peptide inhibitors of caspases (ZVAD, YVAD, DEVD, among others) and Bcl-2 (Newmeyer et al., 1994; Kluck et al., 1997a,b). Additionally, previous work has demonstrated that this system is responsive to proapoptotic signaling molecules such as *Drosophila* Reaper and human caspase-8 (Evans et al., 1997a; Kuwana et al., 1998). Moreover, it has been firmly established that mitochondrial cytochrome c release is critical for apoptosis in this system, as in other systems (Kluck et al., 1997a,b).

In analyzing the requirements for in vitro apoptosis in this system, we and others have previously demonstrated a role for phosphotyrosine signaling pathways in the early events of apoptosis (Liu et al., 1994; Migita et al., 1994; Evans et al., 1997b; Farschon et al., 1997). Building on this, we found that the adaptor protein, Crk, is required for the in vitro apoptosis in this system: immunodepletion of Crk or addition of anti-Crk antisera, rendered the extracts unable to undergo apoptosis (Evans et al., 1997b). Consistent with these results, we found that the isolated Src homology (SH)<sup>1</sup> 2 domain of Crk, but not analogous domains from other adaptor proteins, could inhibit apoptosis, presumably by acting as a dominant negative inhibitor of endogenous Crk-phosphotyrosine signaling interac-

tions. Additionally, depleting extracts of Crk SH2-binding proteins (using the glutathione *S*-transferase [GST]-Crk SH2 domain bound to glutathione-Sepharose) effectively inhibited apoptosis (Evans et al., 1997b).

We report here the surprising finding that the relevant Crk SH2 interacting protein, important for apoptotic signaling in the extract, is the previously well-characterized cell cycle regulator, Wee1 (Nurse and Thuriaux, 1980; Igarashi et al., 1991; Parker et al., 1992; McGowan and Russell, 1993, 1995). Specifically, we have purified Wee1 as a Crk SH2 interactor, demonstrated that Wee1 is required for apoptosis in the extract, and shown that addition of exogenous Wee1 can markedly accelerate apoptosis. Although Wee1 is best known as an inhibitor of the cell cycle kinase, Cdc2, this activity does not seem to be responsible for its apoptotic role, as other similarly potent Cdc2 inhibitors do not enhance apoptotic signaling. Perhaps most importantly, we have found that Wee1's role in apoptosis is linked to Crk signaling. Addition of exogenous Wee1 restored apoptosis to an extract depleted of Crk SH2 interactors, and the ability of Wee1 to accelerate in vitro apoptosis was dependent on the presence of Crk. Collectively, our data suggest that Wee1 and Crk cooperate in a novel pathway of apoptosis in *Xenopus* egg extracts.

## Materials and Methods

### Protein Expression and Reagents

The expression of GST fused to Crk, the Crk SH2 domain, and the R38K mutant of the Crk SH2 domain has been described previously (Evans et al., 1997b). The baculovirus expression clones of GST-Myt1 and His-tagged *Xenopus* Wee1 (XWee1) were kind gifts of Dr. Johannes Rudolph (Department of Biochemistry, Duke University Medical Center) and Dr. William G. Dunphy (Division of Biology, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA), respectively. The expression of Y-3 XWee1 (triple tyrosine mutant; Y90F, Y103F, and Y110F) has been described previously (Murakami et al., 1999). The monoclonal Crk antibody was purchased from Transduction Laboratories. Polyclonal Crk antisera was a generous gift from Dr. Bruce J. Mayer (Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT). Preparation of the crude polyclonal anti-XWee1 antisera has been described previously (Murakami and Vande Woude, 1998). Additional affinity purified polyclonal anti-XWee1 antibody was purchased from Zymed Laboratories. Purified rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories. Antiphosphotyrosine antibody was purchased from Upstate Biotechnology. Glutathione-Sepharose 4B was purchased from Amersham Pharmacia Biotech. Protein A-Sepharose 4B was purchased from Sigma-Aldrich.

### Preparation of *Xenopus* Egg Extracts

For induction of egg laying, mature female frogs were injected with 100 U of pregnant mare serum gonadotropin (Calbiochem) to induce maturation of oocytes. Subsequently, 3–28 d later, a second injection of human chorionic gonadotropin (Upstate Biotechnology) was given to these frogs. Within 12–20 h after human chorionic gonadotropin injection, eggs were harvested for production of extracts. Jelly coats were removed using a 2% cysteine solution (pH 8.0). Eggs were then washed three times using modified Ringer's solution (MMR: 1 M NaCl, 20 mM KCl, 10 mM MgSO<sub>4</sub>, 25 mM CaCl<sub>2</sub>, 5 mM Hepes, pH 7.8, 0.8 mM EDTA), and finally, washed three times in egg lysis buffer (ELB: 250 mM sucrose, 2.5 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, 50 mM KCl, 10 mM Hepes, pH 7.7). Eggs were packed using low-speed centrifugation at 400 g, and subsequently cytochalasin B (5 μg/ml, final concentration; Calbiochem), aprotinin/leupeptin (5 μg/ml, final concentration), and cycloheximide (50 μg/ml, final concentration) were added. Egg lysis was performed using centrifugation at 10,000 g for 15 min. The crude extracts generated from this protocol were supplemented with an energy regenerating system consisting of 2 mM ATP, 5 μg/ml cre-

<sup>1</sup>Abbreviations used in this paper: GST, glutathione *S*-transferase; LC, liquid chromatography; MBT, mid-blastula transition; SH, Src homology; XWee1, *Xenopus* Wee1.

atine kinase, and 20 mM phosphocreatine (final concentrations). Recombinant proteins added to these extracts were diluted into protein concentrating buffer (XB: 100 mM KCl, 0.1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM KOH-Hepes, pH 7.7, and 50 mM sucrose) at the indicated protein concentrations.

For visual apoptosis assays, nuclei were formed in extracts by addition of demembrated sperm chromatin (~2,000 nuclei/μl). Extract samples were taken at various time points during a room temperature incubation and subjected to formaldehyde fixation and staining with Hoechst 33258. Changes in nuclear morphology associated with apoptosis were monitored using fluorescence microscopy.

To prepare purified cytosolic extracts, crude interphase extract was ultracentrifuged at 200,000 g (70 min, 4°C) in a Beckman Coulter TL-100 centrifuge using a TLS 55 rotor. The cytosolic fraction was removed and recentrifuged for an additional 25 min at 200,000 g.

### Purification of Crk SH2-binding Proteins

25 ml of crude egg extract was incubated with 5 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) coupled to GST or GST-Crk SH2 for 30 min at room temperature using the manufacturer's protocol. The beads were pelleted and washed five times with ELB including 1 mM sodium vanadate and combined in a Bio-Rad Laboratories column. The bound proteins were eluted from the resin with 10 ml (5 × 2 ml) boiling SDS-PAGE buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.7 M β-mercaptoethanol). The eluate was loaded into dialysis tubing (8-kD molecular mass cut off) and concentrated by placing Aquacide I (Calbiochem) around the dialysis tubing. When the eluate had been concentrated to 200 μl, it was dialyzed against 62.5 mM Tris, pH 6.8, 0.7 M β-mercaptoethanol overnight, supplemented with bromophenol blue, and loaded on a long 7.5% SDS-PAGE gel. The gel was stained with Pro Blue Colloidal Blue stain (Owl Separation Systems), and relevant protein bands were subjected to peptide microsequencing (as described below).

### Peptide Microsequencing

The bands of interest were excised from the one-dimensional gel and digested in-gel with trypsin (Boehringer) in 10 mM Tris, pH 8.0, according to the procedure of Shevchenko et al. (1996) except that alkylation of sulfhydryls was accomplished with 4-vinylpyridine. After incubation at 37°C for 16 h, digests were vortexed and placed in a sonication bath for 5 min. The liquid around the gel pieces was removed and saved. The gel pieces were then extracted with a second aliquot of 10 mM Tris that was pooled with the extracts. Finally, the gel pieces were extracted with acetonitrile, which was combined with the other extracts. Volume and organic content in the in-gel digest extract was reduced by concentration in a Speed Vac (~60 μl, final volume). Proteolytic peptides from the in-gel digests were analyzed using a fully automated nanoscale capillary liquid chromatography system (Famos autoinjector/Ultimate chromatograph; LC Packings, Inc.) coupled with a hybrid quadrupole/time-of-flight tandem mass spectrometer (Q-ToF; Micromass, Inc.). 50-μl aliquots of the proteolytic peptides in 0.1% formic acid-water were injected by the auto sampler onto a preconcentration/desalting column (300 μm inner diameter [ID] × 1 mm long, 5 μm PepMap C 18; LC Packings, Inc.) at a flow rate of 25 μl/min. After desalting using a mobile phase of 0.1% formic acid-water, the trapped peptides were back-flushed onto a nanoscale capillary liquid chromatography (LC) column (75 μm ID × 15 cm long, 3 μm PepMap C 18; LC Packings, Inc.). A linear mobile phase gradient at 200 nl/min of 2–32% acetonitrile in water (0.1% formic acid in both buffers) over 30 min was used to elute the peptides into the ion source (Z-Spray; Micromass, Inc.) of the mass spectrometer. The electrospray was generated by connecting the LC eluant to nanoelectrospray tips (360 μm outer diameter [OD], 20 μm ID, tapered down to 10 μm OD, 5 μm ID; New Objective, Inc.) which were distally coated with platinum. A 2,000 V potential was applied to the spraytips, giving a stable electrospray across the LC gradient. Data-dependent scanning software (MassLynx; Micromass, Inc.) was used to acquire both molecular weight (MS spectra) and amino acid sequence (production MS/MS spectra) information from the peptides in chromatographic real time. Protein identifications from the dataset were accomplished by using Mascot software (Matrix Sciences) to search a nonredundant database, comparing the experimentally obtained MS data (molecular weight) and MS/MS data (amino acid sequence) with in-silico predictions from the nonredundant database.

### DEVDase Assays

To monitor caspase activity, 3-μl aliquots of each extract sample at various time points were incubated with 90 μl of DEVDase buffer (50 mM Hepes,

pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric peptide substrate Ac-DEVD-pNA (200 mM, final concentration) (BIOMOL Research Labs, Inc.). Enzyme reactions were performed at 37°C for 30–60 min. The absorbance of the colorimetric product was measured at 405 nm using a LabSystems MultiSkan MS microtiter plate reader. As the samples for this assay were analyzed spectrophotometrically, the density of pigment granules in the extracts caused some variation in the background level of absorbance at 405 nm.

### Crk and Wee1 Coimmunoprecipitation

Anti-c-Myc, HA, or Crk monoclonal antibody (2.5 μg antibody/sample) was coupled to protein A-Sepharose beads (Sigma-Aldrich) for 1 h at 4°C and washed in ELB twice. The antibody-coupled beads were blocked to prevent nonspecific protein binding in ELB plus 10 mg/ml BSA for 20 min at 4°C and washed twice with ELB. The isolated cytosolic extract was supplemented with 1 mM sodium vanadate and an ATP-regeneration mix and incubated at room temperature for 30 min. 20 μl of antibody-coupled beads was added to 175 μl cytosolic extract and incubated for 1 h at 4°C. The beads were then pelleted and washed four times with ELB containing 1 mM sodium vanadate. Proteins were eluted from beads by boiling in 2× sample buffer and run on a 7.5% SDS-PAGE gel. The gel was transferred to membrane and probed with polyclonal antisera directed against the XWee1 protein.

This same immunoprecipitation was also performed using affinity-purified polyclonal antisera directed against XWee1 (Zymed Laboratories) coupled to protein A-Sepharose (as described above) or affinity-purified rabbit IgG (Jackson ImmunoResearch Laboratories). Proteins eluted from these beads were separated on a 10.5% SDS-PAGE gel and Western blot analysis was performed using a polyclonal antibody directed against Crk (Transduction Laboratories).

### Immunodepletion and Antibody Addition Assays

Protein A-Sepharose resin (50-μl aliquots) (Sigma-Aldrich) was washed two times with ELB and subsequently incubated with 1% BSA in ELB for 30 min at 4°C. This resin was then washed two additional times with ELB and then incubated with 10 μg of anti-XWee1 IgG (Zymed Laboratories) (control: 10 μg of rabbit IgG; Jackson ImmunoResearch Laboratories) or 50 μg total protein of polyclonal anti-Crk antiserum (control: 50 μg total protein of preimmune serum) in a 1% BSA/ELB solution (total volume 400 μl). Protein A-IgG complexes were formed at 4°C for 1 h and then washed two times in ELB. Egg extract samples (250 μl) were depleted with the protein A-antibody resins (50 μl) two times for 30 min at 4°C. Depleted extracts were supplemented with the energy-regenerating cocktail (described above), incubated at room temperature for 3–5 h, and subjected to a DEVDase activity assay (as described above).

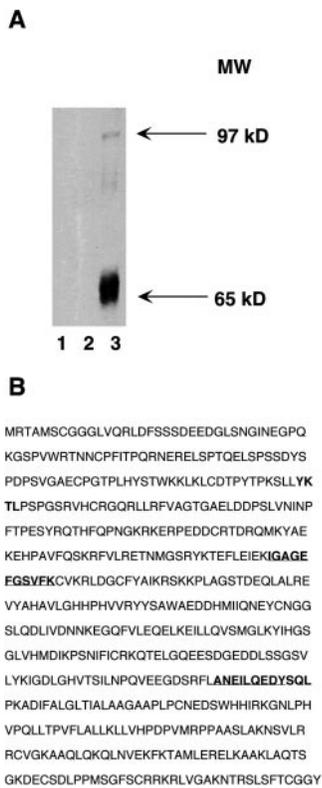
### Crk SH2-binding Protein Pulldown Assays

The GST, Crk wild-type, Crk R38K SH2, or Crk SH2 recombinant proteins were linked to glutathione-Sepharose beads. For pulldown assays analyzed by Western blotting, 25 μl of beads was incubated with 200 μl extract for 30 min at room temperature. The beads were then pelleted and washed three to five times with ELB supplemented with 1 mM sodium vanadate to prevent tyrosine dephosphorylation. The bound proteins were eluted in 2× SDS sample buffer and separated on 7.5% SDS-PAGE gel.

For determining effects of Crk SH2 domain on apoptotic activity, this same technique was used. Extracts were depleted two times on the GST protein resins under the conditions described above. The depleted extracts were then supplemented with the energy-regenerating mix described above and incubated at room temperature for 3–5 h. Samples were taken from these extracts at various time intervals and subjected to a DEVDase assay (as described above).

### Immunoblotting

Immunoblotting was performed after SDS-PAGE and transfer to polyvinylidene fluoride membranes (Millipore). Blots were incubated with appropriate primary antisera described above and subsequently with (secondary antibodies) horseradish peroxidase-linked goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Blots were developed using an enhanced chemiluminescence kit (Renaissance; Dupont).



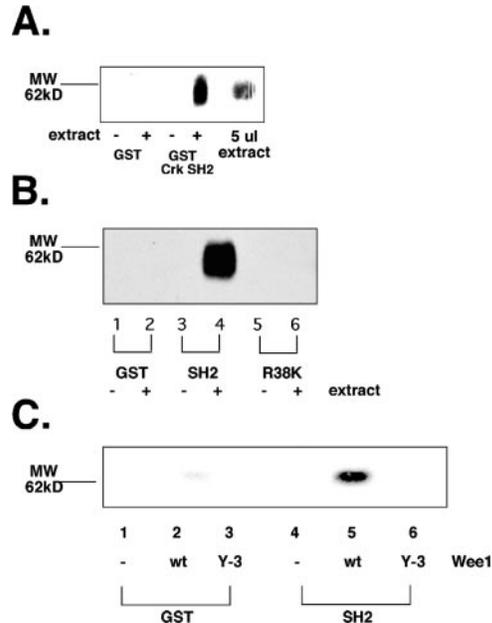
**Figure 1.** Purification of Crk SH2 binding proteins. (A) Crk SH2 binding proteins were isolated as described in Materials and Methods, resolved using SDS-PAGE, and processed for Western blot analysis using an antiphosphotyrosine antibody. Lane 1, GST beads plus extract; lane 2, GST-SH2 beads plus ELB; lane 3, GST-SH2 beads plus extract. (B) Microsequencing analysis identified Wee1 as the specific 68-kD major Crk SH2 binding protein. Mass spectrometric analysis yielded two peptides that match exactly the sequence of the XWee1 protein. The first peptide, IGAGEFGSVFK, corresponds to amino acids 216–226 in the Wee1 protein sequence, and the second peptide obtained from microsequencing, ANEILQEDY, matches amino acids 395–403 (bold and underlined). Putative Crk SH2 binding consensus sequences (YKTL, amino acids 110–113; YSQL, amino acids 403–406) are indicated by bold text.

## Results

### The Crk SH2 Domain Interacts Specifically with the Tyrosine Kinase Wee1 in *Xenopus* Egg Extracts

As we had found that extracts depleted of Crk SH2 interactors failed to undergo apoptosis, we wished to identify Crk binding partners critical for transmission of the apoptotic signal (Evans et al., 1997b). Accordingly, *Xenopus* egg extracts were passed over a column of recombinant GST-Crk SH2 domain cross-linked to CNBr-activated Sepharose. SH2-bound proteins were eluted by boiling in SDS-PAGE buffer, resolved by SDS-PAGE, and, since SH2 domains function through binding phosphotyrosine residues, immunoblotted with antiphosphotyrosine antibodies. As shown in Fig. 1 A, only two tyrosine phosphorylated proteins from the egg extract were detected in association with the Crk SH2 domain.

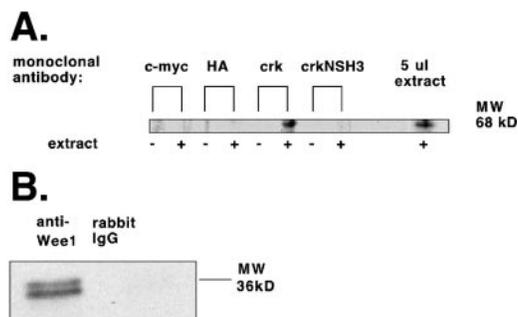
To identify the tyrosine phosphorylated proteins seen binding to the SH2 domain, we scaled up the affinity chromatography procedure and analyzed the 68- and 97-kD tyrosine phosphorylated proteins by nanospray mass spectrometry. Surprisingly, the 68-kD binding protein was unambiguously identified as XWee1. The 97-kD protein was identified on the basis of 10 peptide sequences as vitellogenin A2-precursor protein, a highly abundant protein involved in yolk formation of rapidly maturing oo-



**Figure 2.** Wee1 binds to the Crk SH2 domain. (A) GST-fused Crk SH2 domain linked to glutathione-Sepharose was used to precipitate Crk SH2 binding proteins from egg extracts. GST bound to glutathione-Sepharose resin was used as a control for nonspecific protein binding. Bound proteins were eluted in SDS-PAGE sample buffer and detected by Western blot with polyclonal anti-Wee1 antisera. (B) A mutant form of the Crk SH2 domain (R38K), which does not bind tyrosine phosphorylated proteins, does not bind Wee1. Resins consisting of GST (lane 2), GST-SH2 (lane 4), or GST-SH2R38K (lane 6) bound to glutathione-Sepharose were incubated with *Xenopus* egg extract. The bead-bound material was washed several times with ELB to remove nonspecific protein binding and then resolved by SDS-PAGE. Wee1 binding was detected by immunoblotting with an affinity-purified anti-XWee1 antibody. GST, GST-SH2, and GST-SH2R38K resins that were not incubated with extract (lanes 1, 3, and 5, respectively) were also resolved on this gel as negative controls. (C) Tyrosine phosphorylation of Wee1 is required for its interaction with Crk SH2 domain. Lysates were generated from baculovirus-infected Sf9 cells expressing either wild-type (wt) XWee1 or a mutant form of XWee1 in which three tyrosines (Y-3 Wee1; Y90, Y103, and Y110) were mutated to phenylalanine. These lysates were incubated with either GST (lanes 2 and 3) or GST-Crk SH2 domain (lanes 5 and 6) bound to glutathione-Sepharose. Bead-bound material was washed several times with ELB to remove nonspecific protein binding and subsequently resolved using SDS-PAGE. Western blot analysis, using an affinity-purified polyclonal anti-XWee1 antibody, was used to determine whether XWee1 (lanes 2 and 5) or Y-3 Wee1 (lanes 3 and 6) bound to either of the two resins. As negative controls, GST resin (lane 1) and GST-Crk SH2 resin (lane 4), which were not incubated with extract, were also resolved on this gel.

cytes of oviparous species (vitellogenesis) (Wallace, 1985; Gerber-Huber et al., 1987; Wahli, 1988). Although the 97-kD protein contains potential consensus sites for Crk binding, given its abundance and its previously suggested role in vitellogenesis, we considered Wee1 a more likely candidate for the relevant Crk-binding protein involved in apoptotic signaling.

As shown in Fig. 1 B, the XWee1 sequence contains several previously mapped tyrosine phosphorylation sites that



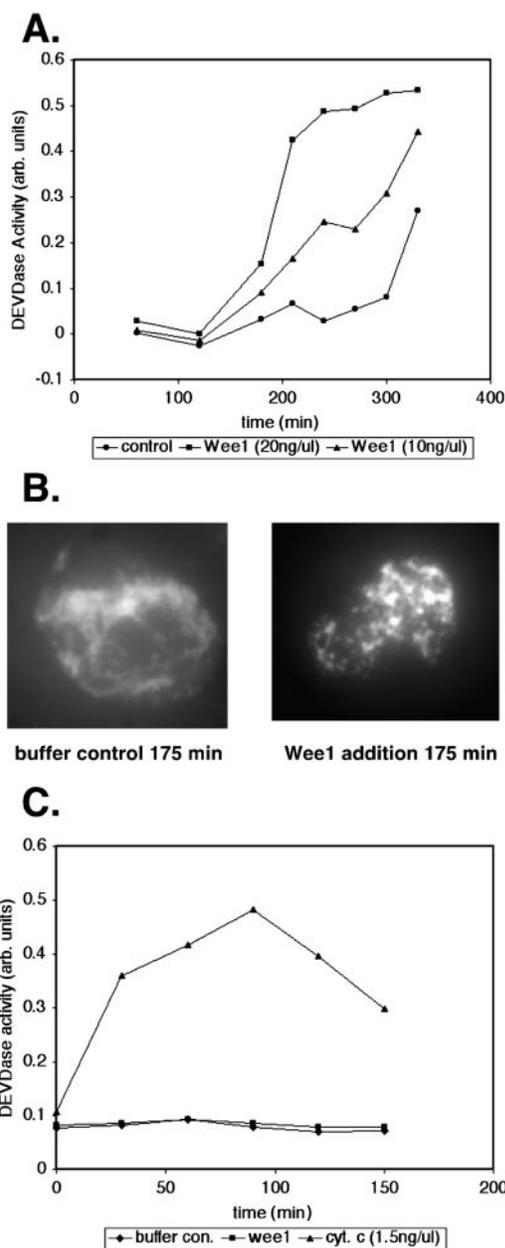
**Figure 3.** Endogenous Crk and Wee1 physically interact in egg extracts. (A) Anti-c-Myc, HA, or Crk monoclonal antibodies were bound to protein A-Sepharose and used to precipitate bound proteins from egg extract as described in Materials and Methods. Bead-bound material was washed in ELB to remove nonspecific protein binding, and then resuspended in SDS-sample buffer and processed for Western blotting using a polyclonal Wee1 antibody for detection. (B) Reciprocal immunoprecipitation was performed using a polyclonal Wee1 antibody or control IgG bound to protein A-Sepharose. Samples were processed for Western blotting using an anti-Crk monoclonal antibody for protein detection.

conform to the consensus for Crk SH2 binding (YXXP/L) (Birge et al., 1993; Songyang et al., 1993). To confirm that Wee1 could bind to the Crk SH2 domain, the population of Crk-binding proteins was again resolved by SDS-PAGE and examined by immunoblotting with anti-Wee1 sera. As anticipated, the anti-XWee1 sera recognized a band of 68 kD in the GST-Crk pulldown, which was absent from control pulldowns with GST alone (Fig. 2 A). Consistent with a role for tyrosine phosphorylation in mediating the SH2-Wee1 interaction, a mutant form of the Crk SH2 domain (R38K; Mayer and Hanafusa, 1990) unable to bind tyrosine phosphorylated substrates was not able to bind Wee1 from egg extracts (Fig. 2 B). In addition, the Crk SH2 domain could not bind a variant of Wee1 mutated so as to change tyrosine to phenylalanine at the known sites of Wee1 tyrosine phosphorylation (Fig. 2 C).

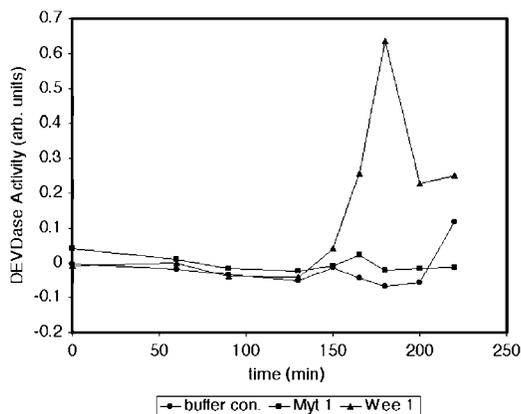
To demonstrate an interaction between endogenous Crk and Wee1 proteins in the egg extract, Crk immunoprecipitates were immunoblotted with polyclonal anti-Wee1 sera. As shown in Fig. 3 A, an anti-Wee1 reactive protein of 68 kD coprecipitated with the Crk monoclonal antibody, but not with control monoclonal antibodies. In the reciprocal experiment, a 34/36-kD doublet of Crk protein, detectable with an anti-Crk monoclonal antibody, was found in anti-Wee1, but not control IgG immunoprecipitates (Fig. 3 B).

### Wee1 Addition Accelerates Apoptosis in *Xenopus* Egg Extracts

As our interest in identifying Crk-binding proteins was focused on proteins involved in apoptotic signaling, we wished to determine whether Wee1 participated in the regulation of apoptosis in egg extracts. Hence, we produced recombinant His-tagged XWee1 in baculovirus-infected Sf9 cells for addition to egg extracts. As shown in Fig. 4 A, addition of exogenous Wee1 produced a marked acceleration of egg extract apoptosis, as measured by caspase activation (monitored spectrophotometrically using a model



**Figure 4.** Addition of exogenous Wee1 accelerates apoptosis in egg extracts. (A) His-tagged XWee1 produced in baculovirus-infected Sf9 cells (20 or 10 ng/ $\mu$ l, final concentration) or XB was added to egg extracts. During a room temperature incubation, extract samples (3  $\mu$ l) were taken at the indicated times and processed for DEVD-pNA cleavage activity. (B) His-tagged Wee1 (20 ng/ $\mu$ l, final concentration; right) or XB (buffer control; left) was added at 1:10 (vol/vol) to extracts supplemented with nuclei ( $\sim$ 2,000 nuclei/ $\mu$ l). Extract samples (2  $\mu$ l) were taken at various time intervals, fixed with formaldehyde, and stained (chromatin) with Hoechst 33258. Fluorescence microscopy was used to visualize the nuclear morphological changes associated with apoptosis. (C) His-Wee1 (20 ng/ $\mu$ l, final concentration), purified cytochrome c (1.5 ng/ $\mu$ l, final concentration), or XB was added to purified cytosolic egg extracts lacking heavy membrane components, including mitochondria, at a 1:10 (vol/vol) dilution. During a room temperature incubation, extract samples were taken at the indicated times and processed for DEVD-pNA cleavage activity.



**Figure 5.** Wee1, but not Myt1, accelerates caspase activation in egg extracts. Recombinant GST-Myt1 and His-Wee1 were diluted into XB such that the amount of Cdc2-phosphorylating activity of each preparation was equal. These kinases, normalized for activity, were then added to egg extracts at a 1:10 dilution (vol/vol). These treated extracts were incubated at room temperature and subjected to a DEVDase assay with extract samples taken at the indicated times.

caspase-3 substrate, DEVD-pNA). Other indicators of apoptotic progression were also accelerated by exogenous Wee1: as shown in Fig. 4 B, addition of Wee1 ( $\sim 10$  ng/ $\mu$ l) to extracts supplemented with nuclei promoted accelerated membrane blebbing, chromatin condensation, and nuclear fragmentation. As has been seen with other stimuli that accelerate apoptosis in the egg extract, the caspase activation and nuclear fragmentation were preceded by mitochondrial cytochrome c release (data not shown). Consistent with a requirement for mitochondrial cytochrome c release, Wee1 could not promote caspase activation in purified cytosolic extracts lacking mitochondria (Fig. 4 C). However, the addition of exogenous cytochrome c activated caspases in identical extracts, demonstrating their apoptotic potential. While the absolute timing of spontaneous apoptosis varies from extract to extract, in all cases recombinant Wee1 hastened the onset of apoptosis.

While these data appear to implicate Wee1 in apoptotic regulation, the tyrosine kinase Wee1 has an already well-defined role in the eukaryotic cell cycle: maintaining the inactive state of Cdc2-cyclin complexes through phosphorylation of tyrosine 15 of Cdc2 (Coleman and Dunphy, 1994). This raised the possibility that Wee1 might accelerate apoptosis by virtue of its ability to suppress cyclin-dependent kinases in the extract. To address this issue, we sought alternative means to suppress Cdk activity in the extract. The dual specificity, membrane-associated kinase Myt1 acts in concert with Wee1 to suppress Cdc2 through phosphorylation of Tyr 15 and Thr 14 (Kornbluth et al., 1994; Mueller et al., 1995). Recombinant baculovirus-produced Myt1, lacking its transmembrane domain, acts like soluble Wee1 to inhibit Cdc2. By measuring phosphorylation of Cdc2-cyclin complexes in vitro, we were able to calibrate our Wee1 and Myt1 preparations so as to obtain equivalent amounts of Cdc2-phosphorylating activity in each preparation (data not shown). Upon addition of each of these preparations to egg extracts, we found that only Wee1 was able to accelerate apoptosis; Myt1 had no such

effect (Fig. 5). In agreement with these results, we found that treatment of extracts with the Cdk inhibitor roscovitine was also unable to accelerate apoptotic progression (data not shown). These data suggest that repression of Cdk activity is unlikely to underlie the ability of Wee1 to accelerate apoptosis in egg extracts.

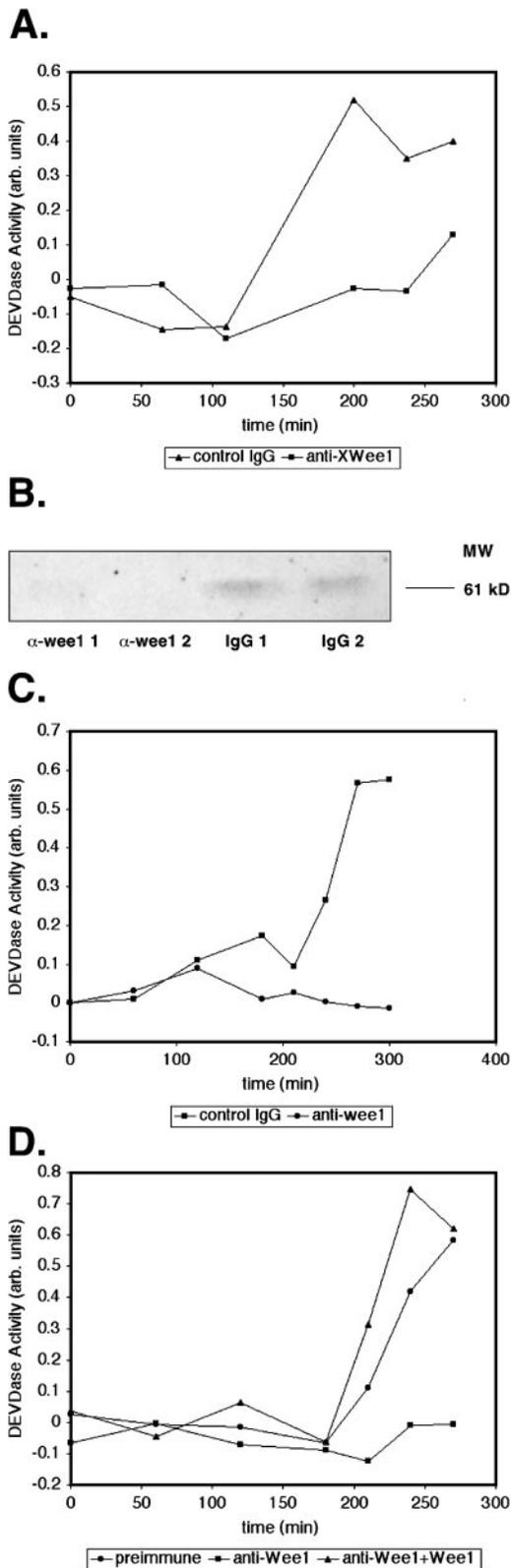
### *Wee1 Is Required for Apoptotic Signaling in Egg Extracts*

Although the ability of exogenous Wee1 to accelerate apoptosis was consistent with a role for Wee1 in apoptotic signaling, it was important to determine whether endogenous Wee1 was required for apoptotic progression of the native extract. As mentioned above, egg extracts incubated at room temperature will, after a variable amount of time (depending upon the particular extract), spontaneously activate caspases. Although the timing is variable from extract to extract, it is highly consistent between aliquots of the same extract. Therefore, we were able to assess the importance of Wee1 for egg extract apoptosis by comparing caspase activation in aliquots of the same extract immunodepleted of Wee1. As shown in Fig. 6, A and B, removal of endogenous Wee1 from egg extracts using a protein A-Sepharose-linked, polyclonal anti-Wee1 antibody caused a significant delay in the kinetics of caspase activation compared with control IgG-depleted extracts. Similarly, the direct addition of Wee1 antibody to the extract (50 ng IgG/ $\mu$ l of extract) inhibited DEVDase activation, whereas control IgG added to extracts at identical concentrations had negligible effects on caspase activation (Fig. 6 C). Furthermore, addition of recombinant Wee1, at concentrations approximating that of endogenous Wee1, could restore caspase activation to extracts treated with anti-Wee1 IgG (Fig. 6 D).

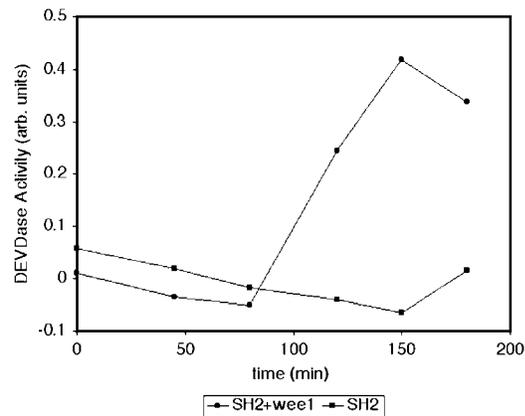
The ability of Wee1 immunodepletion and/or antibody inhibition to strikingly dampen caspase activation was consistent with our hypothesis that removal of Wee1 in association with the Crk SH2 domain was responsible for the apoptotic inhibition that followed depletion of extracts using the GST-Crk resin. Similarly, the ability of free GST-SH2 protein to inhibit apoptosis in the extract presumably resulted from interference with endogenous Wee1-Crk interactions. To prove this more rigorously, we added excess recombinant Wee1 to extracts depleted of GST-SH2 binding partners. As shown in Fig. 7, apoptotic progression, as measured by caspase activation, was restored by addition of Wee1 to the SH2-depleted extracts. In aggregate, these data strongly suggest that Wee1 participates in apoptotic signal transduction in *Xenopus* egg extracts.

### *Wee1 Signals in a Crk-mediated Apoptotic Pathway*

To distinguish whether Wee1-mediated acceleration of apoptosis occurred through Crk or a parallel pathway, we added exogenous Wee1 to extracts immunodepleted of endogenous Crk using Crk antisera bound to protein A-Sepharose. As shown in Fig. 8, Wee1 was considerably more effective at accelerating caspase activation in the presence of endogenous Crk protein. These data suggest either that Crk acts downstream of Wee1 in promoting apoptosis or that Crk and Wee1 must act in concert to signal in apoptotic pathways. As Crk only weakly stimulates ap-



**Figure 6.** Endogenous Wee1 is required for apoptosis in egg extracts. (A) Egg extracts were depleted of endogenous Wee1 using affinity-purified polyclonal anti-Wee1 IgG; or mock depleted using purified rabbit IgG (control) bound to protein A-Sepharose (two consecutive depletions; 4°C, 30 min). Depleted extracts were incubated at room temperature and subjected to a DEVdase activity assay with extract samples taken at the indicated times. (B)



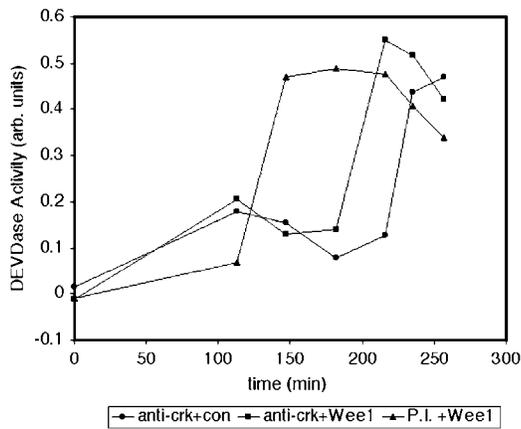
**Figure 7.** Wee1 restores apoptotic activity to Crk SH2-depleted extracts. GST-Crk SH2 domain bound to glutathione-Sepharose was used to deplete egg extracts (two times, 30 min, 4°C). Recombinant Wee1 (20 ng/μl, final concentration) or XB (control) was added to depleted extracts at a 1:10 dilution (vol/vol). The depleted extracts were also supplemented with energy-regenerating cocktail, incubated at room temperature. Samples were taken at the indicated times and subjected to a DEVdase assay.

optosis upon overproduction in egg extracts (Evans et al., 1997b; Smith, J.J., and S. Kornbluth, unpublished results), it may be that binding of Wee1, which would not be in sufficient excess to interact with the majority of the exogenously added Crk, is required in some way to enhance the intrinsic apoptotic activity of Crk.

## Discussion

Although cell-free extracts derived from cultured cells have been instrumental in isolating many core components of the apoptotic machinery (e.g., Apaf-1, cytochrome c, caspase-9; Duan et al., 1996; Liu et al., 1996; Zou et al., 1997), *Xenopus* egg extracts offer a thus far unique opportunity to examine the early signaling events of apoptosis in a biochemically accessible, synchronous system. In this report, we have used this system to examine apoptotic signaling by the Crk adaptor protein and have identified the well-known cell cycle regulator Wee1 as a component of a Crk-mediated apoptotic signaling pathway.

Depletion of endogenous Wee1 was confirmed by Western blot analysis of (2 μl) samples of depleted extracts using the polyclonal Wee1 antibody for protein detection. The numbers 1 and 2 represent the first or second rounds of depletion, respectively. (C) The affinity-purified polyclonal anti-Wee1 IgG or control IgG was added (50 ng IgG/μl extract) at a 1:10 (vol/vol) dilution. These extracts were supplemented with energy-regenerating cocktail, incubated at room temperature, and subjected to a DEVdase activity assay with extract samples taken at the indicated times. (D) Affinity-purified anti-XWee1 IgG or control rabbit IgG was added to egg extracts (final concentration 20 ng/μl). Recombinant Wee1 (final protein concentration 20 ng/μl) or XB was added to the extracts at 1:10 (vol/vol) dilution. These extracts were subsequently treated with an energy-regenerating cocktail and incubated at room temperature. Extract samples (3 μl) were taken at the indicated times and subjected to a DEVdase assay.



**Figure 8.** Wee1 signals on a Crk-mediated apoptotic pathway. Egg extracts were depleted with immune complexes formed from polyclonal anti-Crk antisera or preimmune sera bound to protein A–Sephadex (two consecutive depletions; 4°C, 30 min). Recombinant His-tagged Wee1 (10 ng/μl, final concentration) or XB (control) was added to the depleted extracts at a 1:10 dilution (vol/vol). Extracts were incubated at room temperature, and extract samples were taken at the indicated times in order to assay DEVD-pNA cleavage activity.

### Wee1 Regulates Apoptosis in *Xenopus* Egg Extracts

For each batch of *Xenopus* egg extract, there is a characteristic time that elapses before that extract enters apoptosis. Using many different extracts, we found that addition of exogenous recombinant Wee1 consistently and reproducibly accelerated the initiation of apoptosis, as measured by mitochondrial cytochrome c release, caspase activation, and nuclear fragmentation. Consistent with a role for Wee1 in modulating the *in vitro* apoptosis in *Xenopus* egg extracts, neutralization of Wee1 function through either antibody addition or immunodepletion severely delayed the onset of apoptosis.

Although incubation of egg extracts at room temperature leads to activation of the apoptotic program, the origin of the apoptotic signal in this system is unknown. The ability of these extracts to undergo apoptosis has been speculated to reflect the *in vivo* process of oocyte atresia, wherein oocytes that do not receive the appropriate trophic support die by apoptosis (Hughes and Gorospe, 1991; Smith et al., 1991; Tilly et al., 1992; Newmeyer et al., 1994). An alternative hypothesis is based on the observation that inhibition of zygotic transcription/translation usually initiated at the midblastula transition (MBT) results in massive apoptosis of embryos at the early gastrula transition. (Hensey and Gautier, 1997; Sible et al., 1997; Stack and Newport, 1997). The available data on this phenomenon are consistent with the presence of a maternal apoptotic inhibitor that is degraded over time and must be replaced by synthesis of new (or more) zygotic inhibitors at the MBT to prevent apoptosis when the maternal inhibitor is depleted at the early gastrula transition. It is possible that a maternal apoptotic program is unmasked when maternally encoded inhibitors of apoptosis are degraded or otherwise inactivated by incubation of egg extract on the bench.

In *Xenopus*, Wee1 is present during early oogenesis (stages I–IV), is absent from stage VI oocytes, reappears

at meiosis II, and persists throughout gastrulation (Murakami and Vande Woude, 1998; Nakajo et al., 2000). Therefore, if Wee1 participates in an *in vivo* process of oocyte atresia, it might do so early in oogenesis. Perhaps more plausibly, the amount of Wee1 translated as oocytes transit to maturation might determine the propensity of the mature oocyte/egg to undergo apoptosis before laying and fertilization. Indeed, preliminary data suggest that extracts prepared from stage VI oocytes, lacking Wee1, are refractory to apoptosis *in vitro* (Evans, E.K., and S. Kornbluth, unpublished results).

Although Wee1 plays a preeminent role in regulating entry into mitosis, this function of Wee1 reflects its ability to suppress cyclin-dependent kinases (Parker et al., 1992; McGowan and Russell, 1993; Coleman and Dunphy, 1994; Murakami and Vande Woude, 1998; Murakami et al., 1999; Nakajo et al., 2000; Walter et al., 2000). The role of Cdc2 and its close relative, Cdk2, in modulating apoptosis has been somewhat controversial. Although discrepancies may legitimately be ascribed to cell type differences, active Cdc2 or Cdk2 has been variously reported either to accelerate apoptosis, inhibit apoptosis, be required for apoptosis, or be entirely dispensable for apoptosis (Meikrantz et al., 1994; Norbury et al., 1994; Chen et al., 1995; Ongkeko et al., 1995; Meikrantz and Schlegel, 1996; Yao et al., 1996; Zhou et al., 1998). As suppression of Cdc2 by either Myt1 or roscovitine had no effect on the rate of apoptotic progression in our extracts, we consider it unlikely that simple suppression of Cdc2/Cdk2 activities underlies the ability of Wee1 to accelerate apoptosis. Although ectopic expression of Myt1 in oocytes was reportedly unable to function like Wee1 in suppressing germinal vesicle breakdown, in that case the authors suspected that Myt1 was only weakly active either due to expression levels or progesterone-induced down regulation (Nakajo et al., 2000). In our experiments, Wee1 and Myt1 preparations were added exogenously, obviating expression problems, after calibration for equivalent levels of Cdc2–cyclin B-phosphorylating activity; roscovitine concentrations used were also shown to be entirely effective in suppressing cyclin B–induced activation of histone H1–directed kinase activity (data not shown). Therefore, these experiments point to an alternative mode of action for Wee1. It is possible that Wee1 does double duty as a cell cycle regulator and an apoptotic regulator, ideally situating it as a pivotal participant in the decision to proceed to fertilization and embryogenesis or to enter apoptosis.

### Wee1 Interacts with a Crk-dependent Apoptotic Signaling Pathway

Previous work aimed at elucidating a pathway of apoptotic signaling in *Xenopus* egg extracts implicated the adaptor protein Crk and proteins interacting with its SH2 and NH<sub>2</sub>-terminal SH3 domains (Evans et al., 1997b). Although Wee1 is not a particularly abundant protein in *Xenopus* egg extracts, in affinity chromatography experiments it emerged as a prominent binding partner of the isolated Crk SH2 domain. Moreover, the ability of exogenous Wee1 to accelerate apoptosis depended on the presence of Crk in the extract.

As the name “adaptor” implies, Crk and proteins like it are believed to function in the physical joining, or colocal-

ization, of distinct classes of molecules, in this case tyrosine phosphorylated SH2 binders and polyproline-containing SH3 interactors (Birge et al., 1996). Most commonly, a membrane-bound tyrosine kinase receptor autophosphorylates after ligand engagement, providing a membrane docking site for an adaptor protein, thereby coconcentrating SH3-bound ligands at the membrane. However, in the case of Wee1–Crk, it would seem that a predominantly nuclear protein, Wee1, interacts with Crk to signal apoptosis. Although Crk has been reported to localize predominantly to focal adhesions in intact cells, we and others have also observed a substantial proportion of Crk in the nucleus of cultured cells, both by immunofluorescence and GFP tagging (Matsuda et al., 1993; Smith, J.J., and S. Kornbluth, unpublished results). Moreover, fluorescein-tagged recombinant Crk concentrates in nuclei in the egg extract (Evans, E.K., and S. Kornbluth, unpublished results). Therefore, it is entirely possible that Crk–Wee1 is part of a proapoptotic signal emanating from the nucleus.

In *Xenopus* egg extracts, intact nuclei only form upon addition of exogenous chromatin or nuclei. However, even in the absence of added nuclei, light membranes present in the extract form sheets of annulate lamellae containing nuclear pore complexes which are competent to transport macromolecules; hence, even an extract entirely lacking nuclei contains structures which may accumulate normally nuclear proteins. Interestingly, the basal rate of caspase activation in extracts containing added nuclei substantially exceeded that in extracts lacking nuclei (Smith, J.J., and S. Kornbluth, unpublished results), and Wee1 further accelerated apoptosis in nuclei-containing extracts (data not shown).

An alternative model for Wee1–Crk function, independent of nuclear compartmentalization, is that Crk functions to bring Wee1 in close contact with a Crk SH3 binder; conversely, Wee1 might function to juxtapose Crk and one of its (non-Crk) binding partners. We have found that depletion of Crk SH3 interactors from egg extracts (using a GST–Crk SH3 column) inhibits Wee1-induced acceleration of apoptosis, further implicating downstream Crk-mediated signaling pathways in Wee1-dependent apoptotic pathways (Smith, J.J., and S. Kornbluth, unpublished results). Interestingly, Coomassie blue staining of GST–Crk SH2 interactors (run in parallel with the antiphosphotyrosine Western blot shown in Fig. 1) revealed approximately seven specific proteins associated with the SH2 precipitates (data not shown). This suggests the possibility that Crk–Wee1 may be part of a larger multiprotein complex. However, apart from Wee1, none of the Coomassie blue-stainable proteins were quantitatively depleted from the extract in association with the GST–SH2 resin (Evans, E.K., and S. Kornbluth, unpublished results). Distinguishing between the various models of Crk–Wee1 function will require identification of Crk SH3 ligands, or potentially other Wee1 ligands, which are required for apoptosis. Interestingly, if any of these mechanisms are relevant to very early embryonic development in *Xenopus*, they must be engaged in the beginning of the first embryonic cell cycle, since tyrosine phosphorylation of Wee1, upon which Crk binding depends, disappears after the first cell cycle until after the MBT (Murakami et al., 1999). Alternatively, Wee1 tyrosine phosphorylation may be permissive for engagement of the apoptotic machinery after the MBT.

## Crk–Wee1 and Mammalian Cell Apoptosis

Inevitably, the question arises as to whether the Wee1–Crk complex participates in an apoptotic process unique to *Xenopus* oocytes/eggs/embryos or whether this complex might play a role in mammalian somatic cell apoptosis. While we and others have observed that overexpression of Crk can accelerate apoptosis in intact human tissue culture cells, it is not yet clear if this is a Wee1-regulated event (Parrizas et al., 1997; Smith, J.J., and S. Kornbluth, unpublished results). However, it is attractive to speculate that genotoxic damage might trigger either a Wee1-dependent, checkpoint-mediated cell cycle arrest or a Wee1-regulated apoptotic pathway, depending on the degree of DNA damage. In addition, cell cycle-regulated inhibition of Wee1–Crk complexes may help to prevent the onset of apoptosis when cells are detached from their neighbors and the substratum at the time of entry into mitosis. During interphase, cells that do not have proper matrix or cell–cell interactions will initiate an apoptotic program coined “anoikis” (Frisch and Francis, 1994; Frisch et al., 1996). However, this program is not initiated at each mitosis, despite the fact that cells round up and lose physical contact with both the extracellular matrix and surrounding cells. Wee1 tyrosine phosphorylation, thought to result from autophosphorylation, is likely absent at mitosis when Wee1 is inactive, thereby preventing binding of Crk and engagement of a Wee1–Crk apoptotic pathway. These and other possibilities await future examination of mammalian Wee1, Crk, and potential Wee1–Crk complexes in apoptotic regulation.

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