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 addi-
tion, 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. Or-
gan.) 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 apopto-
sis, or programmed cell death, is crucial to the health of mul-
ticellular organisms (for reviews see Vaux and Korsmeyer,
1999; Green, 2000). Apoptosis is important in a variety of
physiological contexts, including the maintenance of the immune
system, embryological development, and organismal homeo-
stasy. 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 apo-
totic 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 acti-
vated, 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; Zhou et al., 1999). The apoptosome, once activated, induces proteolytic activation of pro-caspase-9, which subsequently cleaves and activates a workhorse of execution, caspase-3. Hence, the mitochondrial release of cytochrome c is a par-
amount site of regulation of programmed cell death, in
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; Kawanaka 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, nucleoli 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; Tilley 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 Bel-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; Kawanaka 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; Migkeit 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)1 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 interactions. 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 Theriaux, 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. Monoclonal Crk antiserum 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 antiserum 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 Pharmaceuticals. 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 h 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% fixed Ringer’s solution (MMR: 1 M NaCl, 20 mM KCl, 10 mM MgSO4, 25 mM CaCl2, 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 MgCl2, 1.0 mM DTT, 50 mM KCl, 10 mM HEPES, pH 7.7). Eggs were packed from frozen solutions. 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 creatine kinase, 10 mM MgCl2, 5 μg/ml phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, and 0.1 μg/ml glucose-6-phosphate dehydrogenase.
alate kinase, and 20 mM phosphorocreatine (final concentrations). Recombi-
nant proteins added to these extracts were diluted into protein concentra-
ting buffer (20 mM HEPES, pH 7.7, 0.1 mM MgCl₂, 10 mM KCl, 10 mM KKH-
Hepes, pH 7.7, and 50 mM sucrose) at the indicated protein concentra-
tions.
For visual apoptosis assays, nuclei were formed in extracts by addition of
demembranated sperm chromatins (~2,000 nucleoli/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 ul-
tracentrifuged 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 proto-
col. 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) boili-
ng 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 (Calbio-
chem) 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-mercapto-
ethanol overnight, supplemented with bromophenol blue, and loaded on a
long 7.5% SDS-PAGE gel. The gel was stained with Pro Blue Colloidol
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 di-
gested in-gel with trypsin (Boehringer) in 10 mM Tris, pH 8.0, according to
the procedure of Shevchenko et al. (1996) except that alklylation of sulhy-
dryls was accomplished with 4-vidipyrindine. 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;
(Famos autoinjector/Ultimate chromatograph; LC Packings, Inc.) coupled
using a fully automated nanoscale capillary liquid chromatography system

Immunodepletion and Antibody Addition Assays
Protein A–Sepharose beads (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 antisera (control: 50 μg total
protein of preimmune serum) in 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 cock-
tail (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 pro-
teins were linked to glutathione-Sepharose beads. For pulldown assay,
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 so-
dium vanadate to prevent tyrosine dephosphorylation. The bound pro-
teins 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 ex-
tracts were then supplemented with the energy-regenerating mix de-
scribed 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 poly-
vinylidene 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 Labo-
atories). Blots were developed using an enhanced chemiluminescence
kit (Renaissance; Dupont).
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., 1997). 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 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.

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. Bead-bound material was washed in ELB to remove 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) 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.
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 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.

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/µl, final concentration) or XB was added to egg extracts. During a room temperature incubation, extract samples (3 µl) were taken at the indicated times and processed for DEVD-pNA cleavage activity. (B) His-tagged Wee1 (20 ng/µl, final concentration; right) or XB (buffer control; left) was added at 1:10 (vol/vol) to extracts supplemented with nuclei (~2,000 nuclei/µl). Extract samples (2 µl) 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/µl, final concentration), purified cytochrome c (1.5 ng/µl, 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.
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/μl 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 to 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-
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.
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 NH2-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-
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 Weel-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 Weel-dependent, checkpoint-mediated cell cycle arrest or a Weel-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 Weel is inactive, thereby preventing binding of Crk and engagement of a Wee1–Crk apoptotic pathway. These and other possibilities await future examination of mammalian Weel, Crk, and potential Wee1–Crk complexes in apoptotic regulation.

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