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13. ABSTRACT *(Maximum 200 words)*

Apoptosis is a cellular suicide program which rids an organism of genetically damaged or superfluous cells. While apoptosis plays a critical role in development, tissue homeostasis and immune regulation, induction of this important cellular process is the mechanism of numerous chemotherapeutic drugs used in the treatment of breast and other cancers. In fact, the resistance of many tumors to chemotherapy often results from the inhibition of apoptosis usually induced in this treatment. Using the Xenopus egg extract in vitro system, we have previously identified crk, an adaptor protein which consists of one SH2 domain and two SH3 domains, as a necessary component in the apoptotic signaling pathway. Furthermore, we have established that when crk binding proteins are removed from the egg extract, apoptosis is inhibited. We have identified the Xenopus wee1 protein, a known regulator of cell cycle kinases, as a specific crk SH2 binding protein using a recombinant crk SH2 affinity column and mass spectroscopic sequencing techniques. Xenopus wee1 binds specifically to the recombinant crk protein and isolated SH2 domain, and also co-immunoprecipitates with the crk protein from the egg extract. This represents the first known direct interaction between an adaptor-type signaling molecule and a cell cycle regulator. The function of Xenopus wee1 in the crk apoptotic signaling pathway is currently under investigation. We hope that this finding may provide one of the links between cell cycle progression and apoptosis.

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Erica K. Evans   9/27/98
PI - Signature   Date

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Introduction

Apoptosis is a process which destroys potentially harmful or nonessential cells in multicellular organisms. Unlike necrotic death which results from cellular injury and induces tissue inflammation, an apoptotic cell is killed without harming its neighbors. In this way, apoptosis specifically eliminates imperfect or unnecessary cells in an autonomous manner while preserving the integrity of the whole organism.

Apoptosis was first described and is most easily recognized by the morphological changes which appear in cells undergoing programmed cell death. The cells commited to death enter an orderly process of destruction. An apoptotic cell loses contact with its neighbors and shrinks due to exclusion of water from the cell. Although the nuclear membrane remains intact, the nucleus itself condenses, and nuclear lamins are degraded (Lazebnik et al., 1995). The chromatin condenses underneath the nuclear membrane and nucleases degrade the cell's DNA. Membrane blebbing is observed as the cell splits up into 'apoptotic bodies' containing nuclear fragments. These apoptotic bodies are quickly phagocytosed by adjacent cells (Wyllie, 1980).

While the morphological changes which accompany apoptosis are easily observed in organisms ranging from the worm C.elegans to human, information has only recently become available detailing some of the biochemical components of the process. Liu et. al. have shown through fractionation of Hela cell extracts, that cytochrome c release from the mitochondria is required for the induction of apoptosis. Upon release from the mitochondria, cytochrome c binds to a protein called Apaf-1 (Zou et.al.) which in turn binds to and activates a member of the caspase family of proteases (Li et. al.). Caspases are central players in the death machinery. The caspase family of proteases are first expressed as proenzymes or zymogens which must be cleaved after internal aspartate residues to induce catalytic activity (Tewari et al., 1995). The activated caspases cleave subsequent family members as well as other cellular substrates whose cleavage or inactivation leads to the physical manifestations of programmed cell death.

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Once a cell releases cytochrome c from the mitochondria and caspases are activated, it is likely commited to the cell death pathway. While recent studies have elucidated the mechanism responsible for the end phases of apoptosis, there is little information available regarding the signaling pathways and factors required to induce cytochrome c release from the mitochondria and subsequent caspase activation. To investigate upstream signaling in the apoptotic program, we have utilized the Xenopus egg extract system.

The Xenopus System

The advantage of using the Xenopus system to study apoptosis lies in the hardy nature of the extracts generated from the unfertilized eggs. The large Xenopus eggs contain a stockpile of components used after fertilization to produce 4000 cells without growth or transcription. The extracts derived from these eggs can support a number of cellular functions such as mitosis, chromosome replication, and apoptosis, that have been extremely difficult or impossible to reproduce in other in vitro systems.

Newmeyer et al. were the first to demonstrate that nuclear events reminiscent of the apoptotic phenotype (e.g. nuclear fragmentation, DNA degradation) can be induced in Xenopus egg extracts. For the remainder of this proposal, these extracts will be called apoptotic. It is likely the apoptotic egg extracts produced from the Xenopus eggs are a result of oocyte atresia. Most extensively studied in mammals, oocyte atresia is a process by which those oocytes not selected for ovulation are systematically resorbed by apoptosis (Hughes and Gorospe, 1991). It seems probable, therefore, that the apoptotic program in the Xenopus eggs is initiated in vivo.

Functional nuclei can be reconstituted in the egg extract by adding demembranated sperm chromatin. Membrane vesicles present in the extract bind to the DNA and fuse to form nuclear structures. These reconstituted nuclei can replicate their DNA, import nuclear proteins through intact pore complexes and, after incubation for a prolonged period of time, undergo the morphological changes observed with programmed cell death such as DNA fragmentation and

nuclear degradation. The biochemical hallmarks of apoptosis such as the release of cytochrome c from the mitochondria and subsequent caspase activation are recapitulated in this system as well.Once the egg extract is made, at least a three to four hour incubation of the nuclei in the extract is necessary before the morphological and biochemical changes associated with apoptosis occur. During this incubation or latent period, it seems one or more cytoplasmic events take place and the extract prepares for nuclear degradation.

The Xenopus egg extracts provide an advantage over tissue culture systems in that cellular factors implicated in the apoptotic process may be directly added to or depleted from the extract, apoptosis occurs synchronously in all nuclei in the extract, and the extract is easily accessible to biochemical analysis. The requisite incubation period preceding the onset of apoptotic morphology provides a valuable opportunity for examination and manipulation of the intracellular processes involved in apoptosis.

Using the Xenopus egg extract system, we have previously shown that the adaptor protein crk, which consists of one SH2 (Src homology 2) domain and two SH3 (Src homology 3) domains is part of the endogenous signaling pathway responsible for apoptosis in the Xenopus egg extract (Evans et.al. 1997). To summarize these data, immunodepletion of crk from the extract as well as addition of anti-crk antisera to the extract abrogates apoptosis. Co-addition of crk antisera and recombinant Xenopus crk restores apoptosis in the extract. Moreover, addition of a fusion protein which consists of GST linked to the isolated SH2 domain of the crk protein inhibits apoptosis suggesting the exogenous SH2 domain is able to bind to and titrate crk SH2 domain binding protein(s) away from functional crk complexes. Furthermore, apoptosis is inhibited when crk SH2 binding proteins are removed from extracts; GST crk SH2 domain is linked to beads, incubated with extract, and removed by pelleting the beads. This suggests we are able to remove factors on crk SH2 beads required for the induction of apoptosis. Similar results are obtained with the isolated N-terminal SH3 domain of crk.

The identification of the SH2 bound factor or factors required for apoptotic signaling has been the focus of my recent studies. Using a crk SH2 affinity column, Xenopus wee1 has been

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identified as a specific crk SH2 binding protein. Wee1 has been previously characterized as a cell cycle regulatory tyrosine kinase responsible for inhibitory phosphorylation of the cyclin dependent kinases (cdks) (Mueller et.al.), however, we show here that wee1 specifically binds to the crk SH2 domain as well as the full length crk protein in the Xenopus egg extract. In addition, wee1 and crk can be co-immunoprecipitated from the egg extracts. Studies to elucidate wee1's role in apoptotic signaling in the egg extracts are currently underway.

Experimental Methods, Assumptions, and Procedures

Preparation of GST-fusion proteins

GST-crk SH2, crk R38K SH2, crk wildtype, grb2 SH2 were cloned into gex expression vectors. The bacterial strain Topp 1 (Stratagene) was transformed with these constructs. Bacteria expressing the recombinant proteins were lysed on ice with 10 mgs lysozyme (Sigma) in 50mM Tris pH 7.5, 10mM KCl, 1mM EDTA, 1mM DTT and 1mM PMSF. Bacterial DNA was digested upon addition of 0.1% Sodium Deoxycholate, 15mM $MgCl_2$, and 0.5mg DNAse 1 (Worthington Biochemical Corp.). Lysates were cleared by centrifugation at 11,000 rpm for 45 minutes in Sorvall SS34,and the supernatant was combined with 2 ml of fresh glutatione sepharose beads (Pharmacia). The beads were rotated at 4°C for 45 minutes, spun in a clinical centrifuge and the supernatant was discarded. The beads were then washed four times with 10mM Hepes pH8, 1mM DTT, and 150 mM NaCl. To prepare soluble GST fusion proteins, the glutathione sepharose beads were incubated 3 x 5 min. in 10 mM Hepes pH 8, 1 mM DTT, and 10 mM glutathione. The eluate was concentrated in Centricon 10 (Amicon).

Preparation of CNBr activated sepharose

CNBr activated sepharose (Pharmacia) was resuspended in 1mM ice cold HCl and washed for 15 min. on a sintered glass filter with 1mM HCl. Soluble gst fusion proteins (2.5mg protein/ml resuspended beads) were dissolved in 0.1M $NaHCO_3$ pH 8.3, and 0.5M NaCl, combined with

resuspended beads and coupled at room temperature for 2 hours. The beads coupled to protein were transfered to blocking agent (1M Tris pH 8.0), incubated for 2 hours at room temperature, and washed alternately in 0.1M NaHCO3 pH 8.3, 0.5M NaCl, and 0.1M Sodium Acetate, 0.5M NaCl three successive times. The coupled beads were stored on ice at 4°C.

Preparation of Xenopus egg extracts

To induce egg laying, mature female frogs were injected with 100 U pregnant mare serum gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3-28 days later) with human chorionic gonadotropin (US Biochemical). Fourteen to twenty hours after injection with hCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine, pH 8.0, washed three times in modified Ringer solution (MMR) (1M NaCl, 20mM KCl, 10 mM $MgSO_4$, 25mM $CaCl_2$, 5 mM HEPES, pH 7.8, 0.8mM EDTA) and then washed in egg lysis buffer (ELB) (250mM sucrose, 2.5mM $MgCl_2$, 1mM DTT, 50mM KCl, 10mM HEPES, pH 7.7). Eggs were lysed by low speed centrifugation at 400 g. Following addition of aprotinin and leupeptin (final concentration 5 ug/ml), cytochalasin B (final concentration 5 ug/ml) and cycloheximide (final concentration 50 ug/ml), eggs were lysed by centrifugation at 10,000 g for 15 min.

Egg Extract Fractionation

To remove mitochondrial components and cleanly separate the cytoplasm and membrane fractions, crude extract was centrifuged at 55,000 rpm (250,000 g) in a Beckman TLS-55 rotor for the TL-100 centrifuge. The cytosolic fraction was removed and recentrifuged at 55,000 rpm for an additional 25 min. Aliquots were frozen in liquid nitrogen and stored at -80°C.

Xenopus wee1 Purification

Twenty mls crude Xenopus egg extract (40mg/ml protein) were incubated with 5 mls CNBr activated sepharose coupled to GST or GST-crk SH2 for 30 min. at room temperature. The beads were pelleted and washed five times with ELB including the phosphatase inhibitor sodium vanadate (1mM) and combined in a Bio-rad column. The bound proteins were eluted off the GST-SH2 resin with 10 ml (5 x 2ml) boiling SDS-PAGE buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, and 0.7M β-mercaptoethanol). The eluate was loaded into dialysis tubing (6-8kD molecular weight cut-off) and concentrated by placing Aquacide (Calbiochem) around the dialysis tubing. When the eluate had been concentrated to 200ul, it was dialyzed against 62.5mM Tris pH 6.8, 0.7M β-mercaptoethanol overnight, supplemented with bromophenol blue and loaded on a long 7.5% SDS-PAGE gel. For microsequencing, the isolated phosphotyrosine containing bands specifically bound to the crk SH2 domain were digested out of the gel with trypsin and sequenced using nanospray technology.

Phosphotyrosine Western blot

SDS-PAGE gels were transferred to PVDF membrane (Immobilon), blocked in 2% BSA in PBS-0.1% Tween 20 overnight at 4°C, incubated with phosphotyrosine monoclonal antibody (UBI) at 1:1000 dilution for 1 hr at room temperature, washed 3 x 10 min in PBS-0.1% Tween 20, and incubated with goat anti-mouse antibody for 45 min. at room temperature. The blot was washed 3 x 10 min. with PBS-0.1% Tween 20 and developed with chemiluminescence reagents (NEN).

Wee1 Western blot

SDS-PAGE gels were transferred to membrane as above and blocked in 5% milk in PBS-0.1%Tween 20 for one hour at room temperature, incubated with anti-wee1 antisera at 1:1000 dilution for 1 hr at room temperature, washed as above, and incubated with goat anti-rabbit antibody for 45 min. at room temperature. The blot was washed and developed as above.

Co-immunoprecipitation

Anti-Wee1 antisera was coupled to Protein A Sepharose beads (Sigma) for 1 hr at 4°C and washed in ELB twice. The extract was incubated at room temperature for 30 min. after which coupled beads were added to the extract and incubated for 1 hour at 4°C. The beads were then pelleted and washed 3 times with ELB containing 0.1% NP-40 and 200mM NaCl. Proteins were eluted from beads by boiling in 2X sample buffer and run on a 12.5% SDS-PAGE gel. The gel was transferred to membrane and probed with a monoclonal antibody directed against the crk protein (Transduction Labs).

Results and Discussion

Several specific crk SH2 domain binding partners isolated from mammalian systems have been previously isolated and characterized. These include the proteins cas (130kD)(Sakai et al., 1994), cbl (120kD), paxillin (70kD) (Sawasdikosol et al., 1995), Shc (56kD) (Matsuda et al., 1994), and IRS-1 (insulin receptor substrate 1, 180kD) (Beitner-Johnson et al., 1996). We obtained antibodies that recognize each of the above proteins from human cells and performed Western blots on Xenopus proteins which are removed from the extract with the crk SH2 beads. Results from these studies were largely inconclusive as only the Shc antibody was capable of recognizing its Xenopus homolog. Xenopus Shc was not detected in the crk SH2 bound material.

Because the antibody studies describe above were uninformative, I decided to use a crk SH2 affinity column for direct isolation of specific binding proteins. As SH2 domains bind phosphotyrosine residues, I used an anti-phosphotyrosine antibody to detect proteins specifically bound to the crk SH2 domain. Two bands, a major band of approximately 65-68kD and a minor band of 97kD specifically associate with the crk SH2 domain (Figure 1). To demonstrate that phosphotyrosine binding capacity of the crk SH2 domain was required to bind these

phosphoproteins, I constructed a recombinant fusion protein consisting of GST linked to a crk SH2 domain containing a point mutation in a conserved region of the SH2 domain (R38K SH2) that abolishes binding to phosphotyrosine residues. As expected, the specific 65kD and 97kD phosphotyrosine containing proteins did not associate with the mutant SH2 domain, as demonstrated by anti-phosphotyrosine Western blot (Figure 1). Importantly, neither the addition of this mutant crk SH2 domain to the extract nor the depletion of mutant SH2 domain binding partners from the extract inhibits apoptosis.

To determine if the phosphotyrosine containing proteins are, in fact, removed from the extract by the crk SH2 domain, the SH2 beads were incubated with extract and pelleted. The "depleted" supernatant was transferred onto fresh SH2 beads and incubated. After incubation with the supernatant, these beads were pelleted and the bound material was blotted with phosphotyrosine antibody. The 65kD and 97kD proteins did not associate with the crk SH2 beads on the second incubation indicating these proteins were depleted by previous incubation with the SH2 beads (Figure 2). As the 65kD and 97kD phosphoproteins require a functional crk SH2 domain for binding and are depleted from the extract by the SH2 domain, those two proteins are the strongest candidates for relevant crk SH2 binding proteins.

In preparation for a large scale purification procedure, the GST crk SH2 domain was covalently coupled to CNBr activated sepharose to minimize loss of the SH2 domain and its binding partners from the beads during purification. The cross-linked beads bind the 65kD and 97kD phosphotyrosine containing proteins (Figure 2) and depletion of the extract on these beads inhibits apoptosis as well as the SH2 domain bound to glutathione sepharose beads, indicating these cross linked beads are capable of binding proteins relevant for apoptotic signaling.

To obtain enough crk SH2 domain binding proteins for identification by microsequencing, 20 mls of extract (concentration 40mg/ml) was incubated in batch with 5 mls of cross-linked SH2 beads at room temperature for 30 min to allow specific binding. The bound proteins were eluted with boiling SDS-sample buffer, concentrated, loaded on a 7.5% long gel for maximal resolution, and stained with Pro-Blue colloidal blue stain. The samples were loaded on the same gel in

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duplicate and processed for an anti-phosphotyrosine Western blot. The tyrosine phosphorylated proteins detected by Western blot were then identified on the gel portion stained with Pro-Blue colloidal blue stain and selected for microsequencing. GST beads incubated in extract or buffer alone, as well as SH2 beads incubated with buffer alone were subjected to the same elution protocol and gel analysis to ensure the extract proteins chosen for sequencing specifically interact with the crk SH2 domain (Figure 3).

Microsequencing analysis of the 65kD major crk SH2 binding protein provided two peptides which matched the Xenopus wee1 protein exactly. 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. Both of these sequences are in the catalytic domain of the wee1 protein and upon BLAST search similar peptides from both the human wee1 and rat wee1 protein are detected. The consensus binding site for the crk SH2 domain was determined by Songyang et al. in a combinatorial peptide screening assay to be phosphorylated tyrosine-X-X-P, or to a lesser extent, phosphorylated tyrosine-X-X-L. Xenopus wee1 does not contain YXXP sequences, however, it does contain two sequences containing a leucine in the +3 position, YKTLPSP (amino acids 110-116), and YSQLP (amino acids 403-407), which closely conform to the defined consensus binding sequence YXXL.

To confirm that wee1, in fact, does bind to the crk SH2 domain, we obtained polyclonal antisera raised against the Xenopus wee1 protein from Dr. George Vande Woude and have performed Western blots on the crk SH2 bead bound material. Wee1 specifically binds the crk SH2 domain and crk wt proteins (Figure 4) but not the mutant R38K crk SH2 domain (data not shown). In addition, the wee1 protein and crk protein are co-immunoprecipitated from the extract suggesting the two proteins associate in the extract (Figure 5). Using this antisera, we have also found wee1 can be immunodepleted from the egg extract, therefore, wee1 will be depleted from the extract and its effects on apoptosis monitored.

To further assess wee1's role in apoptosis, we have obtained a His tagged wee1 baculovirus stock from Dr. Bill Dunphy. Once produced and purified from Sf9 insect cells, wee1

will be added either directly to egg extracts or extracts previously depleted of crk SH2 binding proteins and assayed for apoptosis. If wee1 is a relevant crk binding protein important for apoptotic signaling, it may accelerate apoptosis when added to the extract, or importantly, when supplemented in extracts previously depleted with the crk SH2 domain, the apoptotic phenotype will be restored.

As the analysis of wee1's functional role in apoptosis is being performed, I am also pursuing the identification of the 97kD band which specifically associates with the crk SH2 domain. As the 97kD band seems to bind to the crk SH2 domain with lower affinity or is present at much lower levels in the extract, it will likely require more extract or introduction of a second step purification procedure such as phosphotyrosine antibody resin to isolate enough protein for microsequencing.

Recommendations in relation to the Statement of Work

Technical Objective 1: Identification of those proteins which participate in apoptotic signaling by binding to the SH2 domain of crk. I have completed tasks 1 through 5 which included large scale purification of proteins bound to the crk SH2 domain and am currently completing the functional characterization of isolated proteins in apoptotic signaling (task 7).

Technical Objective 2: Identification of proteins which bind to the crk SH3 glutathione sepharose beads in apoptotic extracts. Because the identification and characterization of the crk SH2 domain binding partners has expanded, I have initiated a collaboration with another member of our lab, Jesse Smith, to identify the crk SH3 binding proteins relevant for apoptotic signaling. This project has revealed a novel SH3 binding protein as well as Sos (son of sevenless), a previously characterized crk SH3 binding partner which acts as an exchange factor for Ras as specific SH3 binding proteins from the egg extract. These studies meet the tasks 2 and 3 and 5 through 9 outlined in my original proposal.

Conclusions

The identification of crk SH2 binding proteins relevant for apoptotic signaling may provide insight into the mechanism of apoptotic induction and, importantly, provide new therapeutic targets for drug development. The Wee1 protein has been characterized as a regulatory kinase which phosphorylates and inhibits the activity of cyclin dependent kinases but has not been previously shown to bind to the crk protein.

There has been significant discussion in the literature as to whether cell cycle progression and programmed cell death are coupled (reviewed in King et. al.) as well as whether the cell cycle kinases such as cdc2 or cdk2 are required for the induction of apoptosis. It is interesting to speculate that crk may function in apoptotic signaling by sequestering wee1 or modifying the activity of wee1 towards these cell cycle kinases. In this way, depletion of wee1 from the extract by the crk SH2 domain may alter the levels of active cdks (cyclin dependent kinases) and this perturbation could be responsible for the inhibition of apoptosis observed in the extract. Because the Xenopus egg extract system is used extensively as a model to study the cell cycle and its cdks, reagents are readily available to test the relationship beween cell cycle progression and apoptosis.

As it has been shown that wee1's role in cdk regulation can be accomplished by another regulatory kinase, myt1, it is also possible that wee1 has other, as yet unidentified, substrates which are important for transduction of the apoptotic signal and removal of this kinase from the extract inhibits propagation of that apoptotic signal.

The elucidation of signaling molecules which induce apoptosis has lagged behind the discovery of the components required for the end stages of apoptosis and destruction of the cell. The initiating signal and the factors necessary to transmit that signal are important drug targets and the discovery of those components continues to be the goal of this project.
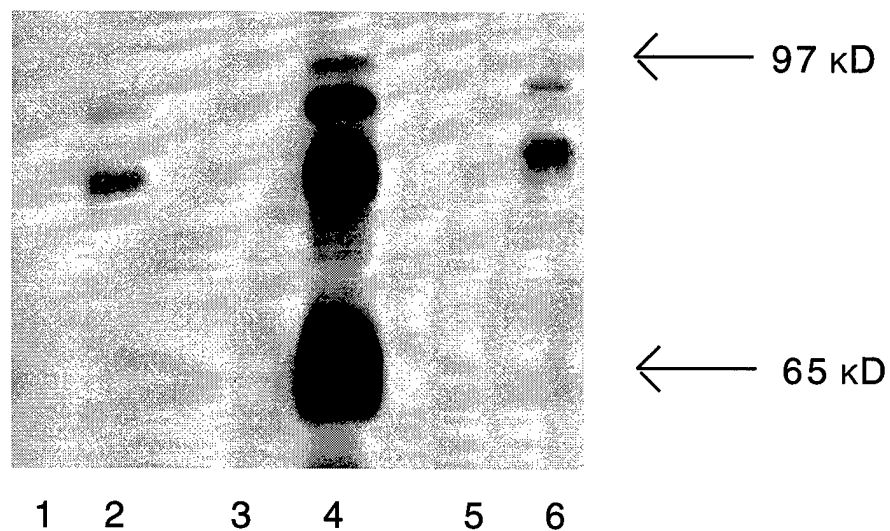
Figure 1. Identification of phosphotyrosine containing crk SH2 binding proteins from Xenopus egg extract. Egg extract was incubated with gst fusion proteins linked to glutathione sepharose beads for 30 min. at room temperature. The beads were pelleted and washed. Bound proteins were eluted and detected by Western blot with phosphotyrosine antibody. The lanes are loaded as follows: 1, gst beads +buffer, 2, gst beads +extract, 3, gst crk SH2 beads +buffer, 4, gst crk SH2 beads +extract, 5, gst R38K crk SH2 + buffer, 6, gst R38K SH2 + extract. The specific binding proteins are denoted by arrows.

GLUTATHIONE SEPHAROSE BEADS

CNBr ACTIVATED SEPHAROSE BEADS

← 97 κD

← 65 κD

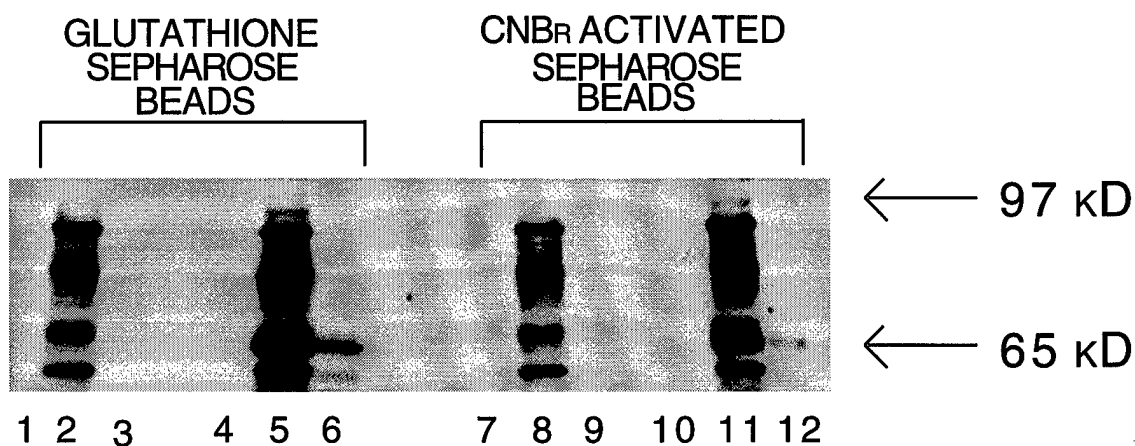1 2 3   4 5 6   7 8 9   10 11 12

Figure 2. Depletion of specific crk SH2 binding proteins from egg extract. Gst or gst crk SH2 was coupled to glutathione sepharose beads (lanes 1-6) or cross-linked to CNBr activated sepharose (lanes 7-12) and incubated with egg extract for 30 min. at room temperature. The beads were pelleted and washed. "Depleted" supernatant was transferred to fresh beads, incubated at room temperature and the beads were pelleted and washed. Bound proteins were detected by anti-phosphotyrosine Western blot. Samples were loaded as follows: lanes 1, gst beads + buffer, lane 2, gst beads + extract, lane 3, gst beads + "depleted" supernatant, lane 4, gst crk SH2 beads + buffer, lane 5, gst crk SH2 beads +extract, and lane 6, gst crk SH2 beads + "depleted" extract. Lanes 1 through 6 and 7-12 , respectively, were loaded in the same sequence. Both the 65kD and 97kD proteins are depleted from the extract by the crk SH2 domain.

A.

200-

97-

67-

43-

1 2 3 4   5

B.

97-

67-

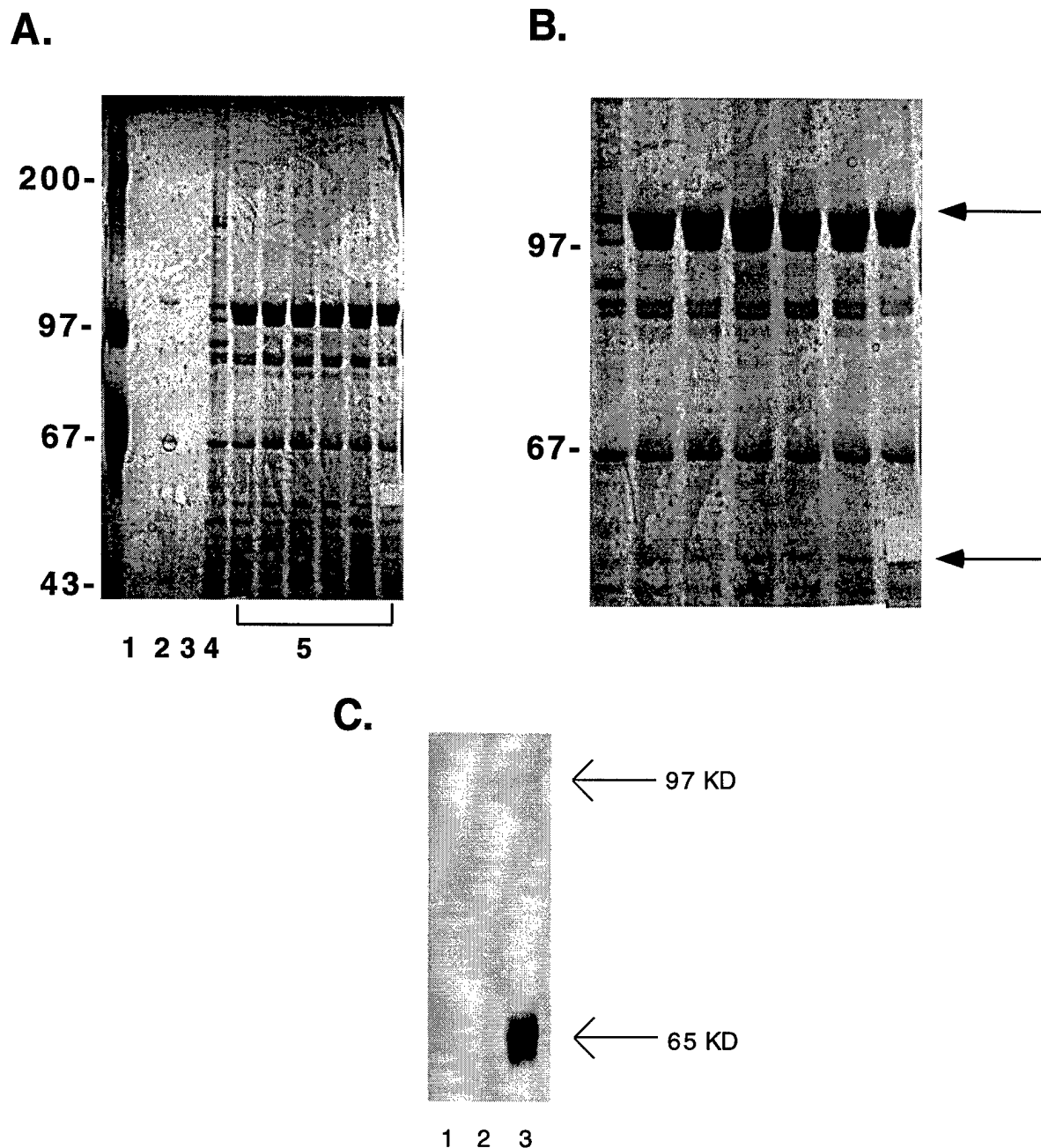**C.**

97 KD

65 KD

1   2   3

Figure 3. Large scale purification of crk SH2 binding proteins. Specific binding proteins were isolated as described in experimental procedures, run on a 7.5% gel and stained with Pro-Blue colloidal stain. A. Pro-Blue stained gel. Lanes are as follows: 1, gst beads +buffer, 2, gst beads +extract, 3, SH2 beads + buffer, 4, SH2 beads + extract (after elution), and 5, SH2 beads +extract (eluate). B. Enlargement of gel from A. with specific binding proteins denoted by arrows. C. Phosphotyrosine Western blot from isolated crk SH2 binding proteins. Lane 1, gst beads + extract, 2, gst SH2 beads + buffer, 3, gst SH2 beads + extract.
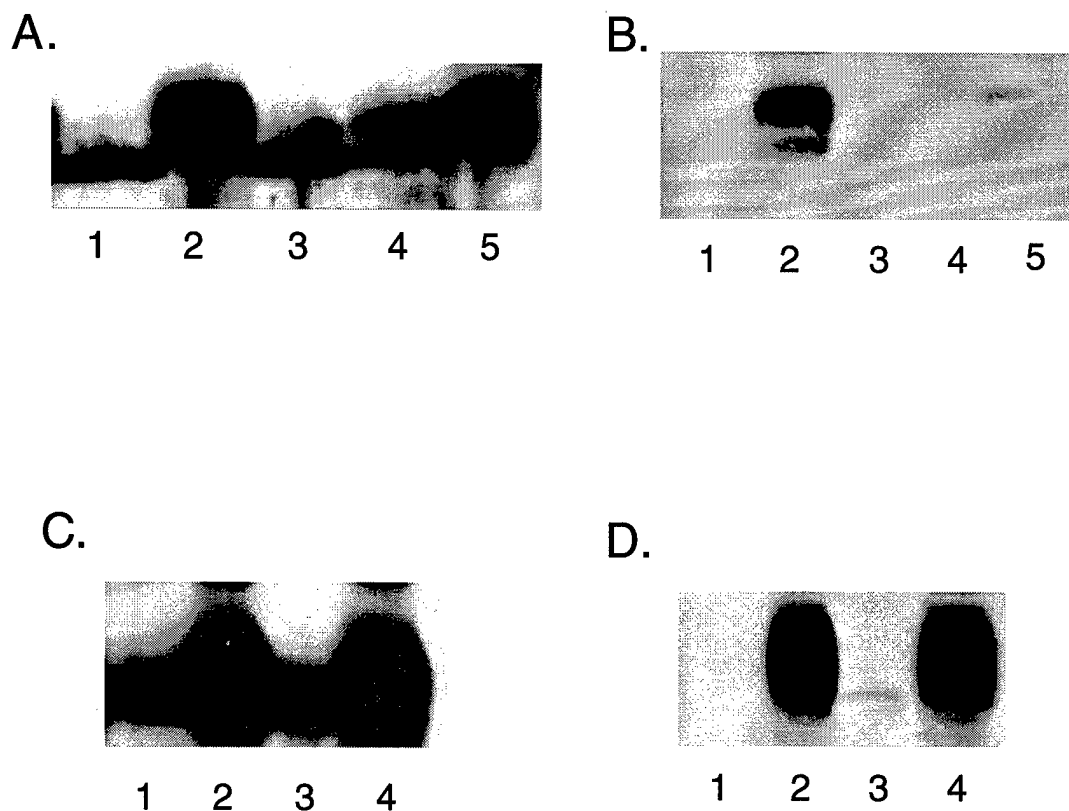
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Figure 4. Specific 65kD crk SH2 binding protein identified as wee1. A. Proteins bound to gst crk SH2 beads +buffer (lane 1), gst crk SH2 beads + extract (lane 2), gst grb2 SH2 beads + buffer (lane 3), and gst grb2 beads + extract (lane 4) were analyzed by Western blot using anti-wee1 antisera. Wee1 from the extract specifically bound to the crk SH2 domain, and bound only slightly to the grb2 SH2 domain. Lane 5 contains extract loaded directly on the gel to serve as a positive control. B. The samples in A. were loaded again and analyzed by anti-phosphotyrosine Western blot to demonstrate wee1 co-migrates with a phosphotyrosine containing protein. Proteins bound to gst crk SH2 beads +buffer (lane 1), gst crk SH2 beads+ extract (lane 2), gst crk wildtype beads +buffer (lane 3), gst crk wildtype beads + extract (lane 4) were analyzed by anti-wee1 Western blot (C), or anti-phosphotyrosine Western blot (D) to demonstrate wee1 binds to full length crk protein in the extract.
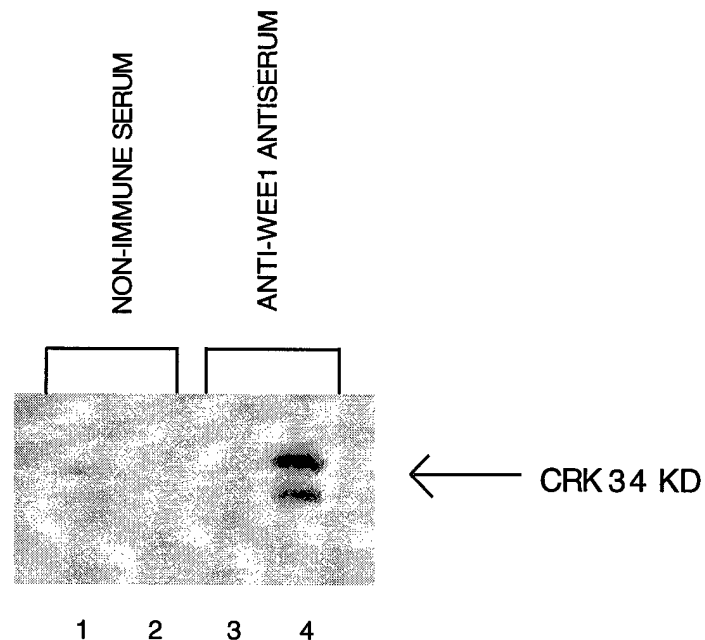
Figure 5. Co-immunoprecipitation of wee1 and crk proteins.
Lane 1, non-immune serum +extract, 2, non-immune serum +
buffer, 3, anti-wee1 serum + buffer, 4, anti-wee1 + extract.
Bead bound proteins were subjected to an anti-crk Western
blot. Crk appears as a doublet (denoted by arrows) with this
monoclonal antibody.

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