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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....11

Reportable Outcomes.....11

Conclusions.....11

References.....12

Appendices.....13

Introduction

A central issue in our understanding of cancer biology is how a tissue or organ maintains the appropriate number of cells. As a normal aspect of animal development and homeostasis, programmed cell death (apoptosis) plays an essential role in maintaining the physiological balance of appropriate cell numbers by opposing uncontrolled cell proliferation. Programmed cell death is controlled and executed by a cell death pathway that is highly conserved from the nematode *C. elegans* to humans. At the heart of this pathway is a family of conserved cell death regulators, first defined by the human anti-apoptotic proto-oncogene *bcl-2*. The Bcl-2 family contains both anti-apoptotic and pro-apoptotic members that may regulate the appropriate activation of apoptosis by interacting with and modulating the activities of other cell death regulators or by affecting the membrane permeability of important organelles such as mitochondria. Abnormal inactivation of apoptosis, such as overexpression of *bcl-2*, can lead to uncontrolled cell growth and contribute to the pathogenesis and progression of various human cancers including breast cancer and tumor resistance to chemo- or radio-therapies. Thus, elucidation of the molecular mechanisms by which Bcl-2 family proteins regulate apoptosis is critical for improving our knowledge of cancer biology. Furthermore, identification of small and potent molecular ligands for Bcl-2 family proteins that can be used to activate or inactivate apoptosis at our will can greatly facilitate the development of new therapeutic methods in the treatment and prevention of breast cancer.

Our objective in this study is to carry out *in vitro* selection (SELEX) to identify high affinity and specificity small RNA ligands (aptamers) for the *C. elegans* cell death inhibitor CED-9 (an invertebrate prototype of Bcl-2 proteins) and three important mammalian Bcl-2 family proteins. We will then use *C. elegans* as a key experimental system and the isolated aptamers to study how CED-9/Bcl-2 family proteins regulate apoptosis and to screen for potent aptamers that potentially can be applied diagnostically or therapeutically in the detection, prevention, or treatment of human cancer.

Three major goals of this study are: 1) Development of the SELEX method and isolation of aptamers for the *C. elegans* cell death inhibitor CED-9; 2) Characterization of CED-9 aptamers and their effects on *C. elegans* cell death; 3) Isolation and characterization of aptamers for mammalian Bcl-2 family proteins and their effects on mammalian cell death.

We have developed an effective SELEX method to isolate aptamers for CED-9/Bcl-2 proteins. We will use aptamers isolated to probe the functional domains of CED-9/Bcl-2 proteins, the interactions of CED-9/Bcl-2 proteins with other cell death regulators, and the mechanistic basis by which CED-9/Bcl-2 family regulate cell death. We will screen for aptamers that can potentially increase or decrease the activity of CED-9/Bcl-2 proteins *in vivo* and characterize the mechanistic and structural basis of such interference by biochemical and structural biological analyses.

The studies described here will provide novel approaches and generate many new useful reagents for studying the mechanisms of Bcl-2 family proteins in apoptosis, which thus far remain poorly understood. But more importantly, these studies may yield simple and powerful diagnostic reagents for the detection of breast cancer and may generate potent apoptosis-inducing compounds and provide important structural insights for designing new therapeutic drugs in the treatment of breast cancer.

Body:

Task 1. Development of the SELEX method and isolation of aptamers for the *C. elegans* cell death inhibitor CED-9

a. Development of an effective and successful SELEX protocol

The graduate student, Jay Parrish, who initiated this project, was close to finishing his Ph.D. study in my laboratory when this grant was awarded. A new postdoctoral fellow, Dr. Chonglin Yang, who has a strong background on RNA work, took over the project when he joined my laboratory in

December 2001. Dr. Yang started the project by testing several strategies (please see below) to improve the SELEX method originally designed by Jay Parrish, who had tried to use this method to isolate aptamers for the SXL protein (*sex* lethal protein), a *Drosophila* splicing factor. Since SELEX is a very sensitive screen, it demands high purity of the target protein to be used in the screen in order to obtain high specificity aptamers. Dr. Yang has spent quite a long time trying to obtain very pure CED-9 protein preparations, using several steps of chromatography purification. He eventually was able to obtain more than 99% pure of two different CED-9 proteins, His₆CED-9(1-251) and His₆CED-9(68-251) (Figure 1). His₆CED-9(1-251) was later used for the Selex experiments.

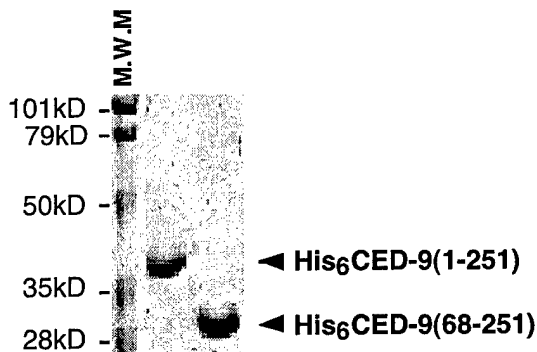


Figure 1. Purified CED-9 proteins. M.W.M. stands for molecular weight marker. 3 μ g of proteins each are resolved on 10% SDS PAGE and stained with coomassie blue.

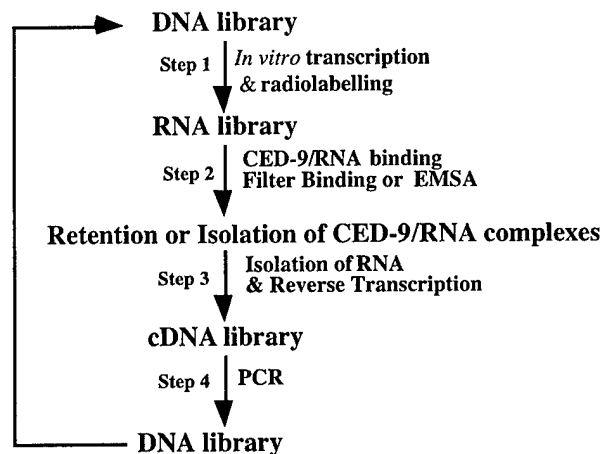


Figure 2 Scheme of SELEX. RNA aptamers are selected on the basis of their ability to bind His₆CED-9(1-251). His₆CED-9(1-251) concentration used is progressively decreased to select high-affinity aptamers.

(step 3), which were then PCR-amplified to generate a new oligonucleotide library enriched in DNAs encoding RNAs with higher binding affinities for CED-9 (step 4, Figure 2).

b. Isolate aptamers for CED-9

Dr. Yang has tried different conditions for CED-9/RNA library binding to minimize the background binding of RNA to the filter membrane. He first gel-purified the RNA library labeled with ³²P and incubated the purified RNA library with slices of filter membrane at room temperature for 30 minutes to remove those RNA molecules that non-specifically bind to the filter membrane. For CED-9/RNA binding, he incubated the above pretreated RNA library with 5 μM His₆CED-9(1-251) (based on a previous pilot experiment). After incubation at 30°C for 30 minutes, the CED-9/RNA mixture was applied to a filter apparatus to isolate CED-9/RNA complexes (Figure 3A). Bound RNA molecules were recovered from the filter membrane by incubation with proteinase K followed by phenol extraction and ethanol precipitation. These RNA molecules were then reverse transcribed into cDNAs and further amplified by PCR. As shown in Figure 3B, RNA molecules bound to His₆CED-9(1-251) were specifically recovered and amplified by RT-PCR. In contrast, few RNA molecules were recovered and amplified by RT-PCR from a control reaction (incubation of purified RNA library with buffer without any protein). Thus we have successfully established a filter-based SELEX protocol that can effectively isolate RNA aptamers from a large pool of RNA molecules (>10¹⁴).

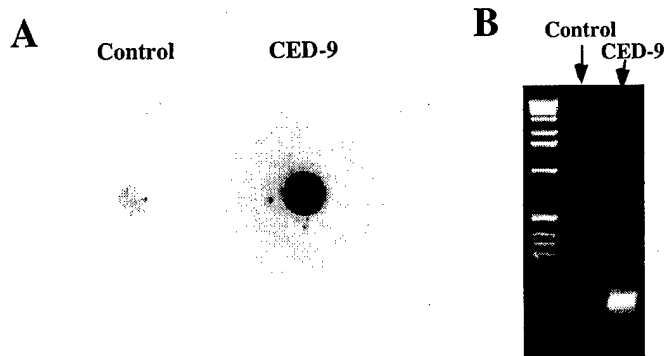


Figure 3. Optimizing protein-RNA binding conditions in the first round of SELEX. A. Filter-binding assay to isolate CED-9/RNA complexes (RNAs were labeled with ³²P). B. Amplification selected RNAs by RT-PCR,

recovered and amplified by RT-PCR from a control reaction (incubation of purified RNA library with buffer without any protein). Thus we have successfully established a filter-based SELEX protocol that can effectively isolate RNA aptamers from a large pool of RNA molecules (>10¹⁴).

We performed 5 more rounds of the filter-based SELEX experiment as described above, with progressively decreasing concentrations of His₆CED-9(1-251) at each round. After 6 rounds of filter-

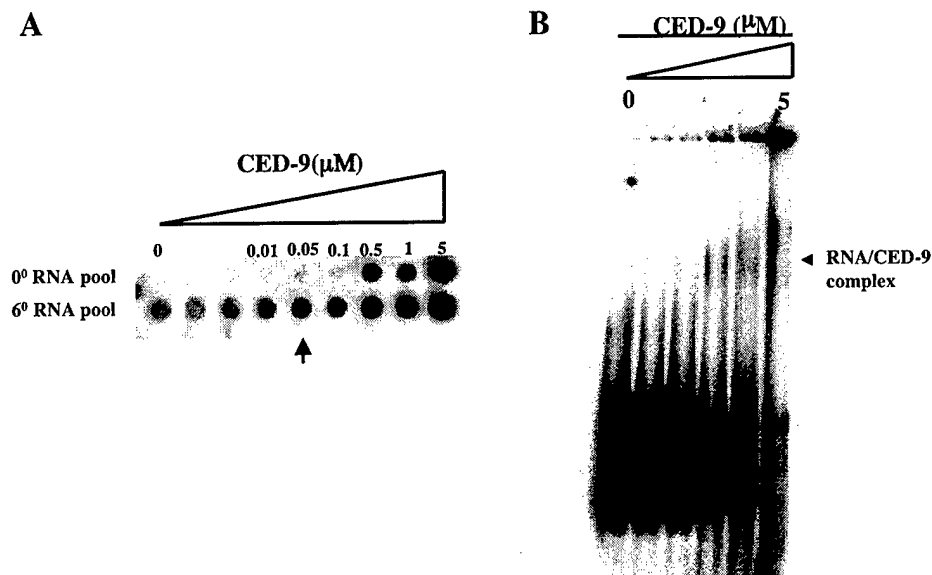


Figure 4. Significantly increased RNA binding affinity to His₆CED-9(1-251) after 6 rounds of SELEX. A. The filter-binding assay. 0⁰ indicates original RNA library. 6⁰ indicates RNAs after 6 rounds of SELEX. B. EMSA of the RNA/CED-9 complex. CED-9 concentrations used were 0, 0.05, 0.1, 0.5, 1, 5 μM from left to right lanes.

based selections, we detected significant increase of binding affinity of the selected RNAs to His₆CED-9(1-251) (Figure 4A). Our data indicated that the selected RNA molecules bound His₆CED-9(1-251) at a protein concentration of 50 nM. In comparison, no RNA/His₆CED-9(1-251) binding was detected at this CED-9 concentration with the original RNA library, confirming that our modified protocol was effective in selecting high binding affinity RNA molecules to CED-9 (Figure 4A).

When the RNA/His₆CED-9(1-251) interaction was examined using the gel mobility shift assay (EMSA) after 6 rounds of filter-based SELEX, RNA/His₆CED-9(1-251) could be detected at a protein concentration of 100 nM, slightly higher than that shown by filter binding (Fig. 4B). To remove those RNA molecules that were selected based on their binding to the filter membrane but not their binding to CED-9, we perform EMSA-based SELEX in later rounds of selections.

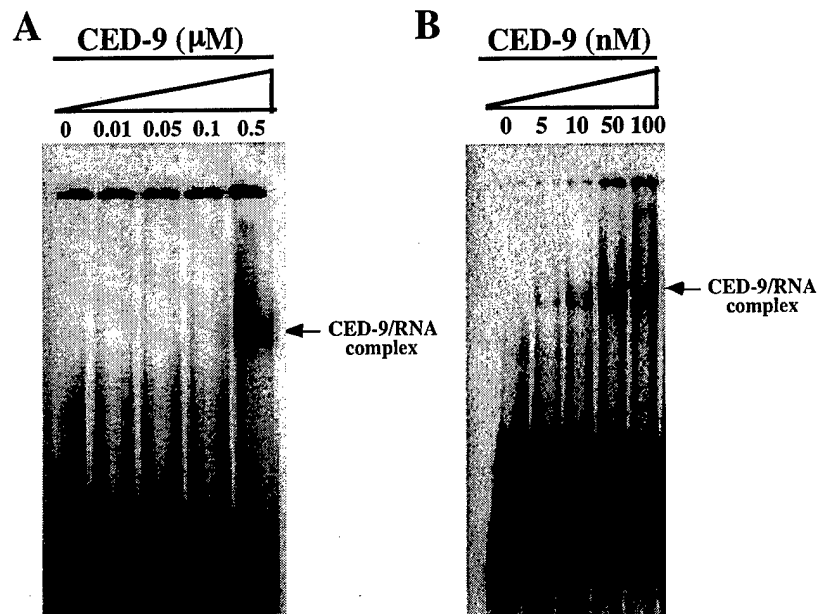


Figure 5. EMSA of CED-9/RNA complexes from round 7 and round 9 SELEX. A, round 7. B, round 9. The concentrations of CED-9 used are indicated.

Shifted RNA/CED-9 bands in EMSA at round 7 SELEX were cut out and treated with proteinase K at 55°C for 1 hour before they were extracted with phenol and precipitated with ethanol. The recovered RNA molecules were amplified by RT-PCR as described above and used for the next round of EMSA-based SELEX. As shown in Fig. 5A (lane 5), after round 7, RNA molecules showed an approximately ten-fold increase in binding affinity to CED-9 compared with those from round 6 (Fig. 4B, lane 6). These RNA molecules were subjected to two more rounds of EMSA-based selections and RNA/His₆CED-9(1-251) interaction could be detected at a protein concentration of 5nM, indicative of significantly improved RNA binding affinity to His₆CED-9(1-251) (Fig. 5B, lane 2).

Thus, using a combination of filter-binding assay and EMSA, we have successfully developed an effective SELEX protocol and have isolated RNA aptamers that bind specifically to the *C. elegans* cell death inhibitor CED-9. The K_d of these RNA aptamers for CED-9 is approximately 10 nM, which is in line with K_ds of aptamers identified in other SELEX studies.

c. Sequencing of aptamers isolated

d. Comparison and analysis of the sequences of aptamers and categorization of the aptamers sequenced.

We are currently sequencing individual CED-9 aptamer cDNA clones to determine their corresponding RNA sequences. We are also in the process of determining the K_ds of individual aptamers for CED-9. The information derived from these analyses will reveal if there are consensus CED-9 aptamer sequences and whether they can be categorized into different subclasses. The sequences of the CED-9 aptamers will also allow prediction of potential secondary and even tertiary structures of these aptamers and how these aptamers may interact with CED-9.

Task 2. Characterization of CED-9 aptamers and their effects on *C. elegans* cell death.

After the sequence analysis of CED-9 aptamers as described above, we will first analyze their K_ds for CED-9 by quantitative binding assays. Aptamers that demonstrate high binding affinities for

CED-9 (1-10 nM Kds), will be further analyzed to identify their binding sites in CED-9. This will be done by GST-fusion protein pull-down assays, using radio-labeled CED-9 aptamers and a serial of CED-9 GST fusion proteins that contain only one specific domain of CED-9 (e.g. the BH1 or the BH2 domain). We expect that a majority of the aptamers isolated may bind to one or multiple BH domains of CED-9 (BH1, BH2, BH3, and BH4) and may thus interfere the interactions of CED-9 with other cell death regulators. For example, BH1, BH2, and BH3 domains form a hydrophobic cleft for the binding of the death-inducing protein EGL-1. We can examine the effects of these aptamers on the binding

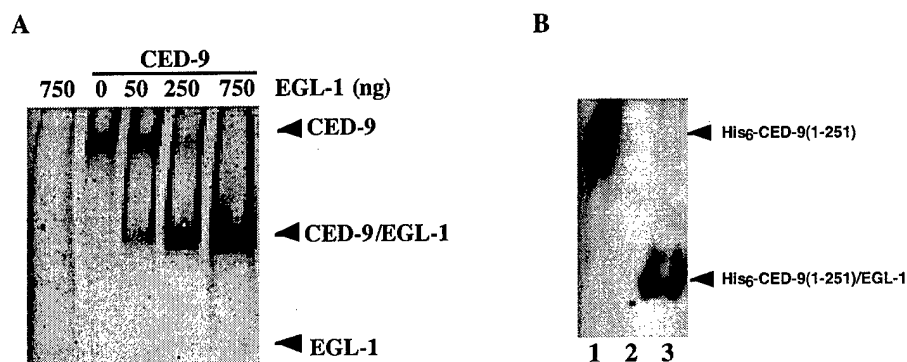


Figure 6 *in vitro* protein interaction assays

A. The CED-9 binding assay. CED-9/EGL-1 complexes (indicated by arrow) can be resolved on native PAGE. Similarly, CED-9/aptamer complexes and the ability of aptamers to disrupt CED-9/EGL-1 complexes can be analyzed by native PAGE.

B. The CED-4 releasing assay. Purified GST-CED-4/His₆CED-9 complexes immobilized on Glutathione beads were treated with buffer (lane 2) or EGL-1 (lane 3) and supernatants were analyzed by native PAGE for the release of CED-9 from the complexes using western analysis and anti-His₆ antibody. Similarly, aptamers that induce CED-4 release from the complexes or that inhibit the release of CED-4 by EGL-1 can be analyzed by this assay.

EGL-1 to CED-9, or EGL-1-induced CED-4 release from CED4/CED-9 complexes, or the binding of CED-4 to CED-9 *in vitro* using two EMSA assays described in Figures 6. The results of these EMSA assays may be used to categorize these aptamers into several interesting classes: 1) Aptamers that can disrupt or block the binding of EGL-1 to CED-9 but do not affect the binding of CED-4 to CED-9. In this case, these

aptamers may function as potent cell-death inhibitors, much like the unusual gain-of function mutation (G169E) in CED-9 does by blocking the binding of EGL-1 to CED-9 but not the binding of CED-4 to CED-9 (Parrish et al., 2000); 2) Aptamers that can disrupt or block the binding of EGL-1 to CED-9, but at the time, mimic EGL-1 to release CED-4 from CED-9. In this case, these aptamers may function as potent cell death inducers like EGL-1 and we can use the EMSAs to select for more potent death inducers than EGL-1 from these aptamers. 3) Aptamers that do not affect the binding of EGL-1 to CED-9 (those that recognize the outside regions of the EGL-1 binding cleft) but prevent EGL-1 from releasing CED-4 from CED-9. These aptamers may also function as potent death inhibitors and will be very valuable reagents to probe the mechanism that mediates the release of CED-4 by EGL-1. 4) Aptamers that do not affect the binding of EGL-1 to CED-9 but disrupt or block the binding of CED-4 to CED-9. These aptamers may function as potent death inducers and may help define the binding sites for CED-4. 5) Aptamers that do not affect the binding of CED-9 with EGL-1 and CED-4 or the EGL-1-induced release of CED-4. In this case, we will still test these aptamers *in vivo* to see whether they have any effect on cell death. If they do, these aptamers may identify functions of CED-9 that do not depend on these protein interactions. These *in vitro* protein interaction analyses in combination with the information on the CED-9 binding sites of these aptamers and analyses and comparison of the sequences and the predicted structures of these aptamers will be very powerful in

elucidating detailed molecular interactions among these cell death regulators and the mechanisms that govern these protein interactions and appropriate life vs. death decisions.

Following the *in vitro* studies of the activities of CED-9 aptamers, those that display interesting *in vitro* activity will be tested for their activities in affecting cell death in *C. elegans*. These aptamers will be expressed in *C. elegans* as extrachromosomal transgenic arrays under the control of a variety of *C. elegans* promoters such as the heat-shock promoters. The expression of the tested aptamers will be confirmed by northern blot analysis and their abilities to promote or inhibit cell deaths in nematodes will be examined by two different quantitative cell death assays. First, we can examine the anterior pharynx of the transgenic animals under the Nomarski microscope and count the number of extra "undead" cells in this region, if the tested aptamers are expected to inhibit cell death. A total of sixteen cells die invariantly in the anterior pharynx of wild-type animals and some or all of these sixteen cells will survive if cell deaths are blocked or inhibited by mutations or inhibitors (Xue and Horvitz, 1995). The number of extra undead cells seen will correlate with the severity of a cell death defect or the strength of death inhibitory activity of an aptamers tested. The second *in vivo* cell death assay involves scoring the number of cell corpses in *C. elegans* embryos in an engulfment mutant background (e.g. a *ced-1* mutant), which results in persistent cell corpse phenotype and thus facilitate scoring of cells that have undergone cell death. Normally, in *ced-1(e1735)* mutant animals, there are around 35 unengulfed cell corpses in late stage embryos. If an aptamer inhibits cell death, then the number of cell corpses in late stage embryos of transgenic animals will be significantly reduced. However, if an aptamer induces cell deaths, then the numbers of cell corpses will be further increased by the expression of the aptamer. Similarly, this assay can be used as quantitative measurement of the potency of death-inhibitory or death-promoting activity of an aptamer. If a specific aptamer cannot be efficiently expressed by a transgenic array, we can directly inject this aptamer into *C. elegans* syncytium gonad and then examine its activity in inducing or inhibiting cell deaths in *C. elegans* germ line, which uses almost the same cell death machinery as that of somatic cell deaths (Gumienny et al., 1999). Correlative analysis of the *in vivo* effects of CED-9 aptamers on cell death and their *in vitro* effects on crucial death regulator interactions not only can verify and improve our mechanistic understanding of the current cell death model but also may lead to new insights on aspects of CED-9 functions that have not been revealed by previous genetic and biochemical studies, especially if some aptamers which do not affect interactions of CED-9 with EGL-1 or CED-4 are found to affect programmed cell death *in vivo*.

The experiments described in this section are either ongoing now or will be executed in the coming fiscal year of the grant. At the end of these studies, we hope that we not only will identify a few CED-9 aptamers that can potently affect apoptosis in *C. elegans* but also can understand the mechanistic basis of their actions.

Task 3. Isolation and characterization of aptamers for other key cell death regulators including mammalian Bcl-2 family proteins

a. Isolation of aptamers for *C. elegans* pro-apoptotic protein, CED-4

CED-4 is required for the activation of programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). Loss-of-function mutations in *ced-4* result in abolishment of all apoptosis in *C. elegans*. CED-4 is homologous to mammalian pro-apoptotic protein, Apaf-1, which complexes with cytochrome c and dATP to activate procaspase9 (Yuan and Horvitz, 1992; Zou et al., 1997). Genetic studies revealed that *ced-4* acts upstream of *ced-3* but downstream of *ced-9* during cell death activation (Shaham and

Horvitz, 1996). Biochemical studies indicated that CED-4 interacts directly with both CED-3 and CED-9 (Chinnaiyan et al., 1997). However, the molecular mechanisms by which CED-4 activates apoptosis are largely unknown. Therefore, understanding of how CED-4 is regulated by CED-9 and how CED-4 functions to activate CED-3 during apoptosis is critical for understanding of how CED-9 acts to regulate apoptosis and will provide important insights into how Bcl-2 family proteins function to regulate apoptosis in general. Although isolation of CED-4 aptamers was not suggested in our original proposal, we decided to go ahead to pursue this experiment.

We have expressed and purified the full-length CED-4 protein from bacteria (Fig.7A). Using

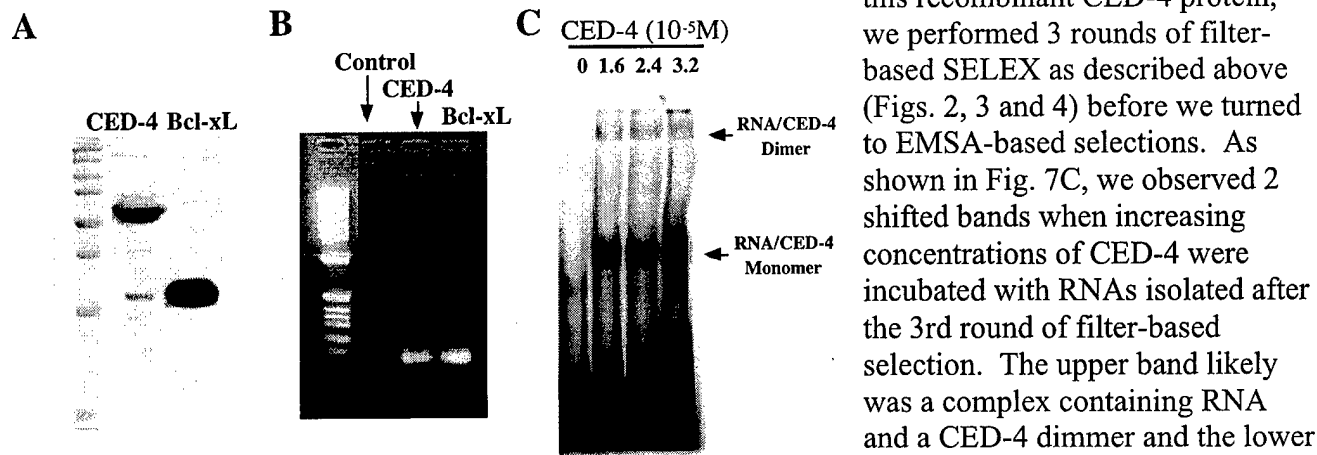


Figure 7. Isolation of aptamers for CED-4 and Bcl-xL. A. Purification of recombinant CED-4 and Bcl-xL. B. Amplification of RNA aptamers for CED-4 and Bcl-xL by RT-PCR (after round 3). C. EMSA of CED4/RNA aptamer complexes.

amplified their corresponding cDNAs by RT-PCR. We will perform 3-4 additional rounds of EMSA-based SELEX with these RNA molecules. Those RNA aptamers with high affinity for CED-4 will be analyzed further for their abilities to modulate the activity of CED-4 using both *in vivo* and *in vitro* assays in the coming fiscal year.

b. Isolation of aptamers for Bcl-xL, one of the major anti-apoptotic proteins in mammalian Bcl-2 family

Mammalian Bcl-2 family contains both pro- and anti-apoptotic proteins (Vander Heiden and Thompson, 1999). Like Bcl-2, Bcl-xL is one of the major anti-apoptotic proteins that are over-expressed in most cancers, including breast cancer (Thompson, 1995). RNA aptamers with high affinity and specificity for Bcl-xL could be used to inhibit the activity of Bcl-xL and sensitize the cancer cells to chemo- or radio- therapies. Eventually these Bcl-xL aptamers may turn out to be useful for diagnosis or treatment of cancers. We overexpressed and purified recombinant Bcl-xL lacking the C-terminus transmembrane region in bacteria (Fig. 7A). Using this protein, we performed 3 rounds of filter-based SELEX and we were able to detect the enrichment of RNAs binding specifically to Bcl-xL by RT-PCR (Fig. 7B). We will next carry out several rounds of EMSA-based SELEX to isolate RNA aptamers with high binding specificity and affinity to Bcl-xL and will conduct functional analysis of these aptamers using both *in vitro* and *in vivo* assays, as described in the original project proposal.

Key Research Accomplishments

- We have successfully purified high quality of CED-9, CED-4 and Bcl-xL proteins for the SELEX screens
- We have developed a very effective SELEX strategy to isolate aptamers, which combines both filter-based and EMSA-based SELEX methods
- We have conducted nine rounds of the SELEX screen for the CED-9 aptamers and have obtained aptamers that bind CED-9 with a Kd of approximately 10nM. We are in the process of sequencing these aptamers and testing their effects on regulating the activity of CED-9 using both *in vitro* and *in vivo* assays.
- We have conducted four rounds of the SELEX screen for CED-4 aptamers and three rounds of the SELEX screen for Bcl-xL aptamers.

Reportable Outcomes

Dr. Chonglin Yang, who originally was a researcher in the field of RNA regulation, is able to switch field to study apoptosis in *C. elegans*, a new model organism and a new research field for him, because of the generous funding of this grant from Department of Defense. He has benefited greatly from such a switch in his training to become an independent scientist. Recently, he was invited to interview for job openings in three prestigious institutes in China (Tsinghua University, Institute of Genetics and Developmental Biology and Institute of Biophysics of the Chinese Academy of Sciences). He also presented a poster at the 14th International *C. elegans* Meeting (Please see appendix). We expect to submit a manuscript describing our studies of CED-9 aptamers within 6-7 months.

Conclusions

We have developed and improved the SELEX method to isolate high affinity and specificity small RNA ligands (aptamers) for a target protein. We have successfully isolated high affinity aptamers for the *C. elegans* cell death inhibitor CED-9 and are in the process of analyzing their effects on the *in vitro* and *in vivo* activities of CED-9. We have also made significant progresses and obtained promising results on our efforts to isolate aptamers for a key *C. elegans* cell death activator CED-4 and aptamers for Bcl-xL, a mammalian homologue of CED-9 and a key mammalian anti-apoptotic protein. Once the aptamers for CED-4 and Bcl-xL are isolated, we will use these aptamers to probe the interactions of CED-9/Bcl-2 with other apoptotic regulators and the functioning mechanisms of CED-9/Bcl-2 family proteins in regulating apoptosis. We will explore the possibilities that some of these aptamers can be used to perturb (induce or block) apoptosis in *C. elegans* and in human cells and thus can be used or modified to become potential diagnostic or even therapeutic agents for the detection or treatment of cancers, which most often are caused by inappropriate apoptosis (Thompson, 1995).

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Appendices

1. An abstract for the 14th International *C. elegans* meeting held at Los Angeles (June 29, 2003 to July 3, 2003)

Program Nr: 639C

Modulation of Programmed Cell Death with RNA Aptamers.

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Programmed cell death (apoptosis) plays an essential role in maintaining the physiological balance of appropriate cell numbers by opposing uncontrolled cell proliferation. The pathway of programmed cell death appears to be highly conserved from *C. elegans* to mammals, suggesting that studies of programmed cell death in *C. elegans* can provide important information for understanding how cell death is regulated and executed in mammals. Moreover, novel ways developed in *C. elegans* to modulate programmed cell death may also be applied to mammals for better detection, prevention as well as treatment of human diseases caused by abnormal apoptosis (e.g. cancer, autoimmune disorders, and neurodegenerative diseases). In this study, we are employing the technique of SELEX (Systematic evolution of ligands by exponential amplification) to identify small RNA aptamers with high binding specificity and affinity for key cell death regulators, including CED-9 and CED-4 from *C. elegans* as well as Bcl-2 and Bcl-xL from humans. We hope to use these RNA aptamers to probe how Bcl-2 family proteins regulate programmed cell death in both *C. elegans* and mammalian cells. More importantly, if these RNA aptamers can be used to successfully modulate the process of programmed cell death in *C. elegans*, they may provide important insights into devising new diagnostic and therapeutic drugs to treat cancer and various apoptosis-related diseases. So far, we have conducted several rounds of SELEX experiments on CED-9, CED-4 and Bcl-xL and have obtained candidate molecules with increasing binding affinity *in vitro* to these proteins. After more rounds of selections, we will investigate in detail the potential effects of candidate RNA aptamers on *in vitro* activities of these key cell death regulators as well as their potential effects on apoptosis in *C. elegans* and in mammalian cells.