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RNA Aptamers Targeting the Cell Death Inhibitor CED-9 Induce Cell Killing in *Caenorhabditis elegans*[*]

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Bcl-2 family proteins include anti- and proapoptotic factors that play important roles in regulating apoptosis in diverse species. Identification of compounds that can modulate the activities of Bcl-2 family proteins will facilitate development of drugs for treatment of apoptosis-related human diseases. We used an *in vitro* selection method named systematic evolution of ligands by exponential enrichment (SELEX) to isolate RNA aptamers that bind the *Caenorhabditis elegans* Bcl-2 homolog CED-9 with high affinity and specificity and tested whether these aptamers modulate programmed cell death in *C. elegans*. Five CED-9 aptamers were isolated and classified into three groups based on their predicted secondary structures. Biochemical analyses indicated that two of these aptamers, R9-2 and R9-7, and EGL-1, an endogenous CED-9-binding proapoptotic protein, bound to distinct regions of CED-9. However, these two aptamers shared overlapping CED-9 binding sites with CED-4, another CED-9-binding proapoptotic factor. Importantly ectopic expression of these two aptamers in touch receptor neurons induced efficient killing of these neurons largely in a CED-3 caspase-dependent manner. These findings suggest that RNA aptamers can be used to modulate programmed cell death *in vivo* and can potentially be used to develop drugs to treat human diseases caused by abnormal apoptosis.

Apoptosis is an essential cellular process that is critical for tissue homeostasis and animal development in metazoans. Abnormal inactivation of apoptosis can result in uncontrolled cell growth, leading to development of cancer and autoimmune disorders. By contrast, inappropriate activation of apoptosis can cause too much cell death, leading to neurodegenerative diseases and immunodeficiency (1, 2). Development of effective therapeutic methods that can correct or reverse inappropriate apoptosis is thus a critical issue in clinical medicine.

Apoptosis is controlled and executed by an evolutionarily conserved cell death pathway (3, 4). At the center of this pathway is a family of conserved cell death regulators first defined by the human proto-oncogene *bcl-2*, which promotes cell survival and was identified by virtue of its overexpression in a number of B-cell lymphomas (5–8). Subsequently a family of Bcl-2-related proteins, characterized by the presence of at least one of four conserved Bcl-2 homology (BH)[3] domains, has been discovered and found in organisms as distantly related as *Caenorhabditis elegans* and humans (8). Members of this family can be either antiapoptotic or proapoptotic and can form heterodimers with selected family members to affect apoptosis. The mechanisms by which Bcl-2 family proteins regulate cell death appear to be quite complicated but likely involve modulation of the mitochondrial permeability and the release of crucial apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor, and endonuclease G, which promote activation of caspases, the cell death executors, and other cell death events such as chromosome fragmentation (8, 9). In addition to B-cell lymphomas, Bcl-2 family members are overexpressed in a wide variety of cancers, contributing to malignant growth of tumors as well as tumor resistance to chemotherapies (10). Thus Bcl-2 family proteins are ideal targets for pharmaceutical intervention in the treatment of cancer and other human diseases.

Genetic studies in *C. elegans* have identified a central cell killing pathway involving four genes (*egl-1*, *ced-9*, *ced-4*, and *ced-3*) that act in a negative regulatory cascade to control activation of programmed cell death (4). Biochemical studies indicate that EGL-1, a BH3-only proapoptotic protein, induces cell death by binding to and inhibiting the activity of CED-9, a cell death inhibitor and a homologue of human Bcl-2, leading to the disassociation of CED-4 from the CED-4/CED-9 complex tethered on the surface of mitochondria (4). CED-4, a homologue of the human apoptotic protease activating factor 1, then directly facilitates the activation of the CED-3 caspase and apoptosis (4, 11, 12). As in humans, misregulation of apoptosis in *C. elegans* can have detrimental outcomes. For example, loss-of-function mutations in the *ced-9* gene cause embryonic lethality as a result of too much cell death (13, 14). Importantly key components of this cell death pathway are highly conserved from nematodes to humans (4), indicating that studies of apoptosis in *C. elegans* will be highly relevant to studies of apoptosis in humans.

Small RNA molecules play critical roles in regulating many important cellular events such as ribosome biogenesis, RNA splicing, gene silencing (RNA interference), and protein translation (microRNA) (15) and can potentially be used as therapeutic agents. In the early nineties, an *in vitro* selection method named systematic evolution of ligands by exponential enrichment (SELEX) was developed to isolate small RNA molecules (aptamers) that have high binding affinity and specificity to important biomolecules such as proteins and RNAs (16, 17). By binding specifically to a region or a domain of their targets, aptamers can directly modulate the biological activities of their targets at the protein level. Most attractively, aptamers have been used successfully to treat human diseases caused by misexpression or altered activity or function of their target proteins. For example, aptamers with high affinity for vascular endothelial growth factor are being clinically used to treat blindness caused by macular dysfunction (18, 19). Aptamers targeting blood coagulation factors VIIa and IXa are promising anticoagulants (20, 21). In addition, aptamers have also been developed to modulate the activities of other proteins critical for various biological processes, including tran-

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[3] The abbreviations used are: BH, Bcl-2 homology; SELEX, systematic evolution of ligands by exponential enrichment; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; PLM, posterior lateral microtubule.

TABLE 1
CED-9 aptamers selected from the RNA library

Clone	Aptamer sequence[a]	Frequency[b]
Group A		
R8-20	GGGTGGTCGCTTATCCGCATAGAGGTTTACGACTTCGGAGACTGCCGATA	3 of 30
R9-7	GGATGGACGCTTATCCGCATAGAGGTTTACTACTTCGGAGACTGCCGATA	9 of 30
R9-4	GGAGTCATGGCGCATAGGTAGCTTGTATGCTGCCAGAGACTGCCCTGTGA	8 of 30
Group B		
R9-2	GGGTGCTTCGAGCGTAGGAAGAAAGCCGGGGGCTGCAGATAATGTATAGC	2 of 30
Group C		
R9-8	GGTTGCCACGTTTATGTACAGAGACCGCCTCGGGAATATGACGCGCAGTA	5 of 30

[a] The aptamer sequences shown are cDNA sequences corresponding to the 49-nucleotide variable region of the RNA aptamers, which were selected from a random RNA library. All RNA molecules in this library contain the following sequence: (T7)AGGGAGGACGATGCGN_{49}CAGACGACGGA. T7 indicates the T7 promoter sequence (AATACGACTCACTATAG). N_{49} designates the 49 random nucleotides in the variable region of the RNA library.
[b] Frequency indicates that the number of clones showing the same AciI digestion pattern among 30 randomly selected cDNA clones from the eighth or ninth rounds.

scription factors NF-κB (22–24) and E2F (25), epidermal growth factor (26), *Drosophila* nuclear splicing factor B52 (27, 28), and others (29–32). Thus SELEX could be an effective method to identify new small molecule regulators for Bcl-2 family proteins.

In this study, we used SELEX to identify RNA aptamers for the *C. elegans* cell death inhibitor CED-9 and isolated five different CED-9 aptamers, which can be categorized into three different groups based on their predicted secondary structures. We found that two of these aptamers, R9-2 and R9-7, and EGL-1 bind to different regions of CED-9 but seem to have overlapping CED-9 binding sites with CED-4. Importantly overexpression of these two CED-9 aptamers induced ectopic cell killing in *C. elegans* touch receptor neurons, and this ectopic killing was suppressed by a strong loss-of-function mutation in the *ced-3* gene. Therefore, these aptamers may antagonize the prosurvival activity of CED-9 and kill cells through the normal programmed cell death pathway. Our results suggest that RNA aptamers for key cell death regulators can yield, or serve as leads to generate, potent compounds to modulate apoptosis *in vivo*.

MATERIALS AND METHODS

Recombinant Proteins—CED-9(1–251)-His_6, which contains all four Bcl-2 homology motifs (BH1, BH2, BH3, and BH4) and binds EGL-1 and CED-4 as well as the full-length CED-9 protein (12, 31), was expressed in bacterial BL21(DE3) cells and purified with nickel-nitrilotriacetic acid-agarose beads according to the instructions of the supplier (Qiagen). After purification (Fig. 1*A*), the recombinant proteins were dialyzed in a buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10% glycerol. Recombinant GST-EGL-1, GST-CED-9 (wild type and mutants), and CED-4 were purified as described previously (11).

SELEX—The RNA library for the first round of SELEX was generated from a DNA template containing 49 randomized nucleotides flanked by fixed linker regions and a T7 promoter for *in vitro* transcription (Table 1). RNA was transcribed overnight and purified using 10% denatured polyacrylamide gels. For the first six rounds of selection, a filter binding assay was used. Briefly RNA was preincubated with a nitrocellulose filter (HAWP, 0.45 μm, Millipore) for 1 h at room temperature to reduce nonspecific binding of RNA molecules. Then the counterselected RNA was incubated with CED-9(1–251)-His_6 at 30 °C for 30 min in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 50 mM NaCl, and 10% glycerol. The reaction mixture was subsequently filtered gently through the nitrocellulose membrane with a filtration apparatus, and the membrane was washed four times with 1 ml of washing buffer (20 mM HEPES-KOH (pH 8.0), 0.2 mM EDTA, 100 mM KCl, and 20% glycerol). The membrane containing the protein-RNA complex was incubated with proteinase K at 55 °C for 20 min in a buffer containing 100 mM Tris-HCl (pH 8.0), 12.5 mM EDTA, 150 mM NaCl, and 1% SDS followed by phenol:chloroform extraction and precipitation with cold ethanol. The amounts of protein and RNA used for binding were gradually decreased with the increasing rounds of selection. An electrophoretic mobility shift assay was used for the last three rounds of SELEX. Approximately 300 cpm ^{32}P-labeled RNA was incubated with different amounts of CED-9(1–251)-His_6 in a reaction mixture of 20 μl for 30 min at 30 °C in the same protein-RNA binding buffer as above and separated with 7.5% native polyacrylamide gels. The shifted protein-RNA complex was cut off the gel, and the RNA was recovered as described above. For both selection assays, the selected RNA was reverse transcribed into single-stranded cDNA with avian myeloblastosis virus reverse transcriptase at 42 °C for 1 h according to the instructions from the provider (Fisher BioReagents). The cDNA was further amplified by PCR using the primers corresponding to the fixed regions at both ends, and the amplified DNA was subjected to the next round of *in vitro* transcription and selection. The RNA selected after round 8 and round 9 was amplified by reverse transcription PCR and ligated into the *C. elegans* expression vector pPD52.102 via its NheI and EcoRV sites. After transformation of the ligation products into DH5α cells, PCR was performed on the resulting colonies to amplify the inserts, which were digested with the 4-base cutter enzyme AciI for fingerprinting on 4% agarose gel. The binding of individual aptamers to CED-9 was further confirmed by gel shift assays, and aptamers with high binding affinity to CED-9 were sequenced. The secondary structures of these aptamers were predicted with the program Mfold (33).

Electrophoretic Mobility Shift Assay (EMSA)—CED-9(1–251)-His_6 or GST-CED-9 protein was incubated with ^{32}P-labeled aptamers (~150 cpm) in a 20-μl reaction mixture in the binding buffer (as described above) supplemented with 2 μg of yeast tRNA as a nonspecific binding competitor. After incubation at 30 °C for 30 min, the reaction mixture was separated on a 7.5% native polyacrylamide gel at 4 °C. For the competition binding assay, the indicated amounts of unlabeled aptamer were added to a reaction mixture with a fixed concentration (0.5 μM) of CED-9(1–251)-His_6. Both ^{32}P-labeled and unlabeled (cold) aptamers were denatured at 85 °C for 5 min and cooled on ice for 5 min before being added to the reactions.

Overexpression of Aptamers in C. elegans—The cDNAs encoding the aptamers with high binding affinity to CED-9 were inserted into the *C. elegans* expression vector pPD52.102 via its NheI and EcoRV sites that directs the expression of the aptamers in six touch receptor neurons under the control of the promoter of the *mec-7* gene. The aptamer expression vectors were linearized with EcoRV and injected into wild-type animals with the co-injection marker pRF4, which causes the Roller phenotype. Two to three independent transgenic lines were scored for cell killing caused by the overexpression of each aptamer.

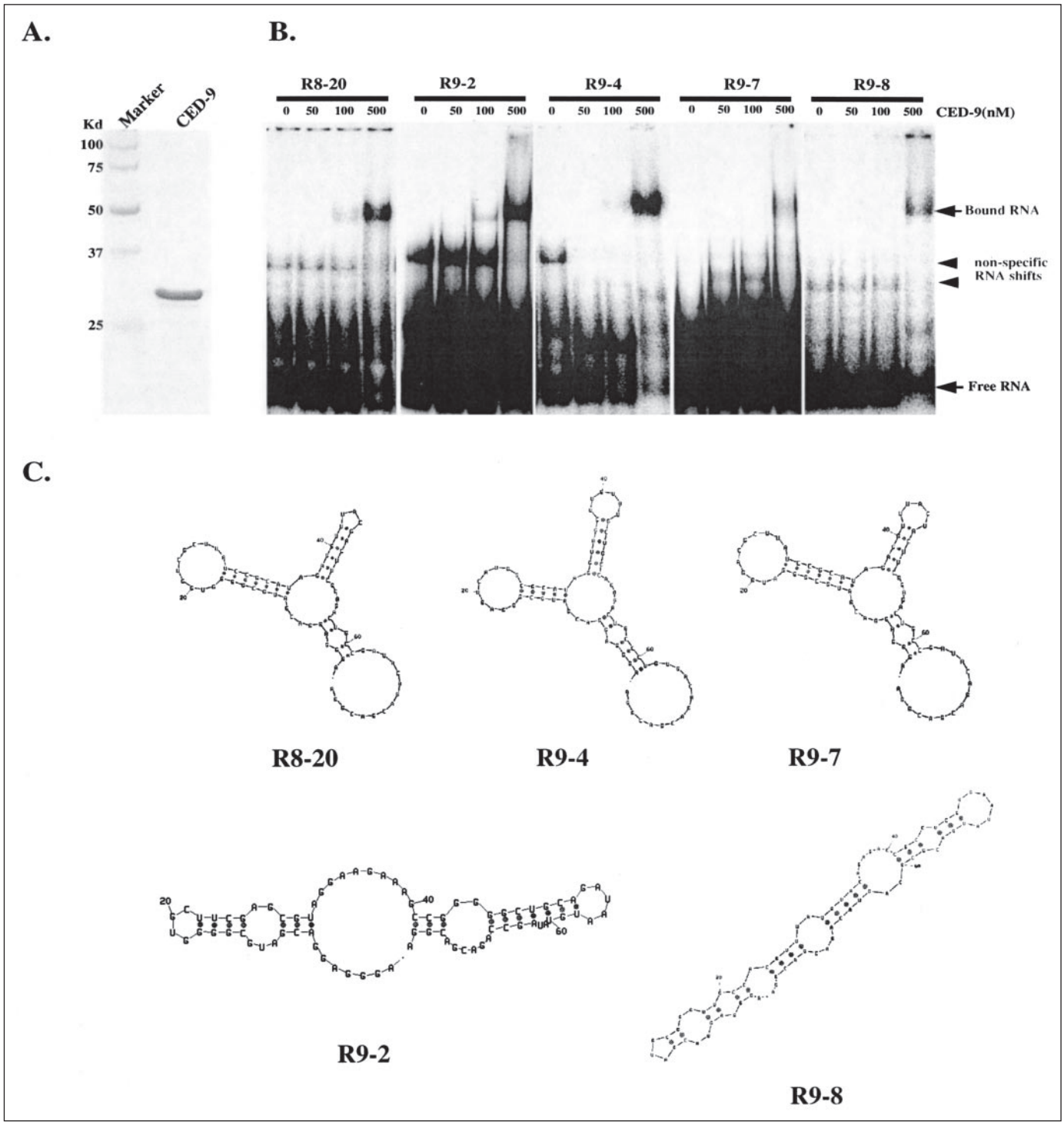

FIGURE 1. **Characterization of CED-9 aptamers.** *A*, a Coomassie Blue-stained gel showing the recombinant CED-9(1–251)-His_6 protein used for SELEX. *B*, binding of aptamers to CED-9. ^{32}P-Labeled aptamer (~300 cpm) was incubated with increasing amounts of CED-9(1–250)-His_6 as indicated at 30 °C for 30 min and resolved on 7.5% native polyacrylamide gels. *Arrows* indicate unbound RNA and bound RNA. *Arrowheads* indicate nonspecific shifts of RNAs, which likely have different secondary structures from the major RNA aptamer species. *C*, predicted secondary structures of CED-9 aptamers.

RESULTS

Isolation and Characterization of CED-9 Aptamers—To identify RNA aptamers for CED-9, SELEX was carried out using a protocol described by Chen *et al.* (26). Briefly an RNA library was generated *in vitro* using an oligonucleotide library that contains a central region of 49 randomized nucleotides flanked at both ends by constant sequences and a bacterial T7 promoter for *in vitro* transcription (Table 1). For the first round of SELEX ~10^{15} unique sequences were represented. Each round of SELEX consisted of the following steps. Radioactive labeled RNAs were incubated with purified recombinant CED-9 before the reaction mixtures were applied to a protein binding assay such as the EMSA or the filter binding assay. CED-9/RNA complexes that were isolated from the EMSA or retained by the filter ("Materials and Methods") were recovered and reverse transcribed to cDNAs, which were

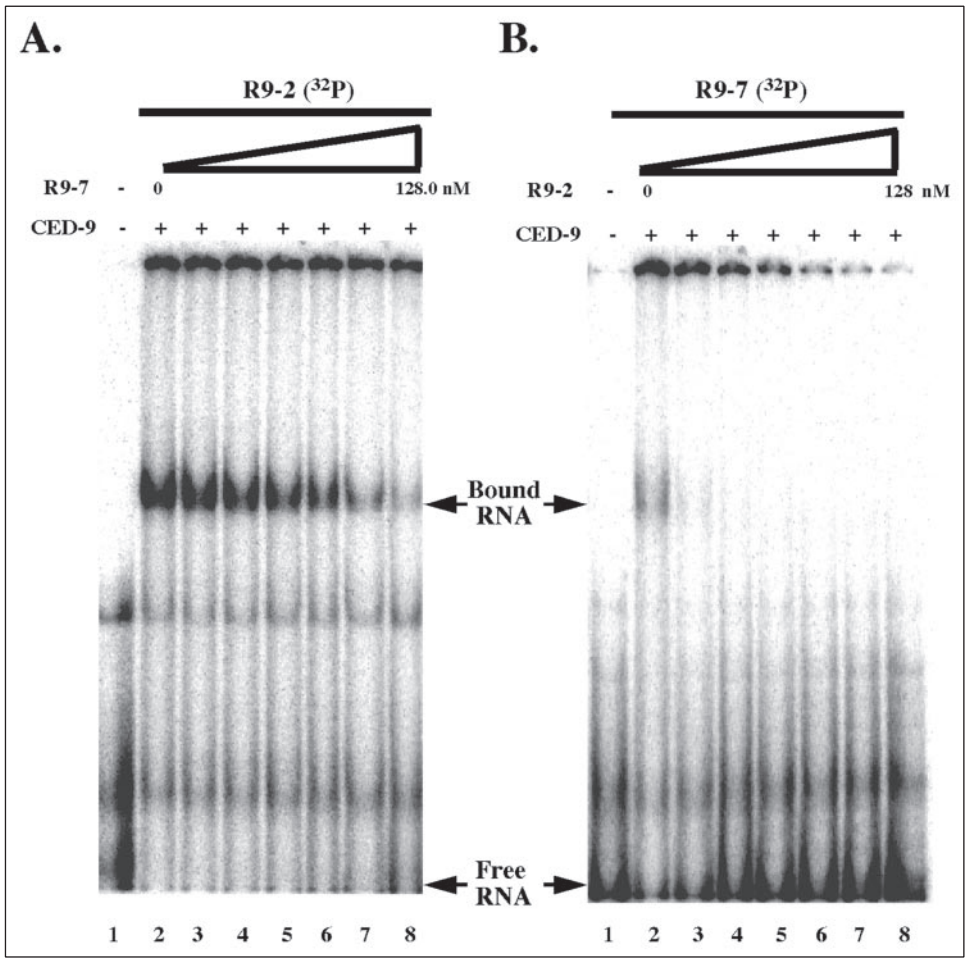

FIGURE 2. **Aptamers R9-2 and R9-7 compete with each other for binding to CED-9.** *A*, approximately 8 nM ^{32}P-labeled R9-2 was incubated with CED-9(1–250)-His_6 (500 nM) in the presence of increasing concentrations (4, 8, 16, 32, 64, and 128 nM) of unlabeled R9-7 at 30 °C for 30 min and separated with a 7.5% native polyacrylamide gel. *B*, approximately 8 nM ^{32}P-labeled R9-7 competes with increasing concentrations (4, 8, 16, 32, 64, and 128 nM) of unlabeled R9-2 for binding to CED-9.

then PCR-amplified to generate a new oligonucleotide library enriched in DNAs encoding RNAs with higher binding affinity for CED-9. After nine rounds of SELEX, we obtained a pool of RNA molecules that bound CED-9 with high affinity (see below).

To determine the sequences of isolated CED-9 aptamers, we cloned the corresponding cDNA molecules of aptamers obtained from the last two rounds of SELEX into a vector. We then checked these cloned cDNA molecules with restriction enzyme fingerprinting to determine the relative abundance of closely related aptamers in these pools ("Materials and Methods"). To do this, the RNA coding regions of 30 cDNA clones from each round of SELEX were PCR-amplified and digested with a frequent 4-base cutter, AciI. Clones with the same restriction digestion pattern were assumed to have identical or very close DNA sequences. Using this analysis, we identified seven different RNA species among the 30 cDNA clones selected from the ninth round RNA pool. From the last two rounds of SELEX, we identified a total of 12 different aptamers that bind CED-9. We further tested their binding affinities for CED-9 using EMSAs and chose the aptamers that bound CED-9 with the highest affinity for further analysis. Five aptamers obtained from these secondary selections are shown in Fig. 1 and Table 1.

Among the five aptamers, R8-20 was isolated from the round 8 SELEX, and the other four were isolated from round 9. Based on restriction enzyme fingerprinting analysis, these aptamers are likely the most abundant RNAs in the last two rounds of SELEX. Therefore further rounds of selection would not likely yield significantly different pools of aptamers. Two of the five aptamers, R8-20 and R9-7, differ by only 3 nucleotides (Table 1). Because these two aptamers were isolated from consecutive rounds of selection, our SELEX method appears to be able to enrich for specific binders of CED-9 as shown by increased frequency of their isolations (3 of 30 for R8-20 and 9 of 30 for R9-7, respectively; Table 1). EMSAs indicated that all these aptamers bound CED-9 very well (Fig. 1*B*). Interestingly R9-2 had slightly higher CED-9 binding affinity than the other aptamers, although the frequency of R9-2 isolation was not as high as those of other aptamers (Fig. 1*B* and Table 1). It is likely that R9-2 will be enriched with more rounds of selection.

To further characterize these CED-9 aptamers, we used the Mfold program to predict secondary structures of these aptamers. Based on the predictions, these five aptamers can be categorized into three groups. Group A includes R8-20, R9-7, and R9-4, which have very similar trifoliolate stem-loop structures. Group B has R9-2 and Group C includes R9-8, both of which contain rodlike stem-loop structures (Fig. 1*C*). The different predicted secondary structures of these aptamers suggest that they may have different CED-9 binding properties. Again the similarity of secondary structures within each aptamer group indicates that our SELEX enriched specific aptamers for CED-9. In the studies described below, we chose R9-7 from group A that is relatively more abundant and R9-2 from group B that has very good binding affinity to CED-9 for more detailed analyses.

R9-2 and R9-7 Aptamers Bind to Overlapping Sites on CED-9—We first determined the binding affinity of R9-2 or R9-7 to CED-9 using competition-based EMSA. In these assays, ^{32}P-labeled aptamer/CED-9 complexes were incubated with increasing amounts of unlabeled aptamer, and the amounts of labeled aptamer that remained in aptamer/CED-9 complexes were monitored. The disassociation constant (K_d) of

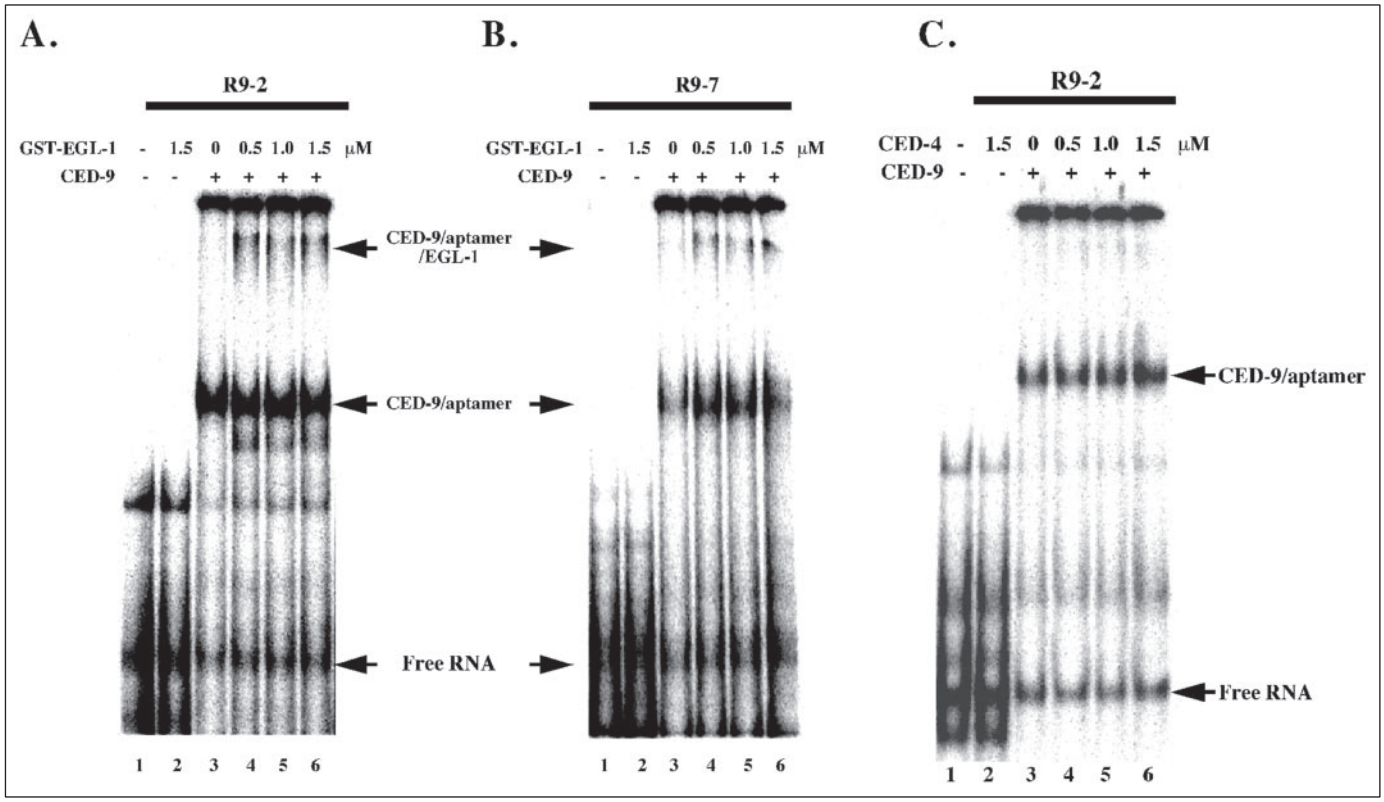

FIGURE 3. **EGL-1 but not CED-4 forms a ternary complex with CED-9 and its aptamers.** *A*, ^{32}P-labeled R9-2 was incubated with CED-9(1–250)-His_6 (500 nM) in the presence of increasing concentrations (0, 0.5, 1.0, and 1.5 μM, *lanes 3–6*) of GST-EGL-1. The reactions were resolved by EMSA. *B*, R9-7 forms a ternary complex with CED-9 and EGL-1 as assayed in *A*. *C*, R9-2 binding to CED-9 in the presence of increasing concentrations of CED-4 as assayed in *A*.

CED-9/R9-2 complexes measured using this assay was ~4 nM, whereas the K_d for R9-7/CED-9 complexes was ~16 nM (data not shown). Based on the Mfold predictions, R9-2 and R9-7 have different secondary structures and may bind to different surface regions of CED-9. We therefore tested whether these two aptamers bind to the same surface regions of CED-9 by performing a modified competition EMSA in which unlabeled R9-7 was incubated with ^{32}P-labeled R9-2/CED-9 complexes or vice versa. As shown in Fig. 2*A*, R9-7 could compete with R9-2 for binding to CED-9, although at least 4-fold excess of cold R9-7 was required for this competition. Similarly R9-2 could efficiently compete away the binding between R9-7 and CED-9, but much less cold R9-2 was needed for the competition (Fig. 2*B*). These results suggest that R9-2 and R9-7 may contact CED-9 at overlapping sites. However, it is also possible that the binding of one aptamer to another CED-9/aptamer complex could induce a conformational change in CED-9 that causes dissociation of the other aptamer. In such a scenario, the two aptamers could bind to different regions of CED-9 but still effectively compete with each other for CED-9 binding. To test this possibility, we performed limited proteolytic digestion of CED-9 or CED-9/aptamer complex with trypsin and monitored the digestion patterns using gel electrophoresis. The CED-9 trypsin digestion patterns were essentially the same with or without aptamers (data not shown), suggesting that no obvious CED-9 conformational change was induced by the binding of the aptamers. In contrast, binding of EGL-1 to CED-9 induces conformational changes of CED-9 (11) and results in a different trypsin proteolysis pattern of CED-9 (data not shown). Taken together, these observations suggest that these two aptamers bind to the same or overlapping regions on CED-9 despite having different predicted secondary structures.

The Effects of Aptamers on CED-9/EGL-1 and CED-9/CED-4 Complexes—In *C. elegans*, CED-9 functions as an antiapoptotic factor. It has been suggested that CED-9 inhibits cell death by binding to and tethering the proapoptotic protein CED-4 onto the surface of mitochondria (34–36). In cells that are doomed to die, the cell death initiator EGL-1 is transcriptionally up-regulated and then binds to CED-9, resulting in the release of CED-4 from the CED-9/CED-4 complex, which then promotes the activation of the CED-3 caspase (11, 12, 36, 37). To evaluate the potential effects of CED-9 aptamers on cell death, we tested whether CED-9 aptamers interfere with the interactions of CED-9 with EGL-1 and CED-4. Using EMSA, we found that EGL-1 could form a ternary complex with CED-9/R9-2 or CED-9/R9-7 as indicated by the supershifted CED-9/aptamer complexes following addition of EGL-1 (Fig. 3, *A* and *B*). Because EGL-1 alone did not interact with either of these aptamers, these results suggest that EGL-1 and R9-2 (or R9-7) likely bind to different regions of CED-9. In contrast, recombinant CED-4 failed to supershift the CED-9/aptamer complexes (Fig. 3*C*), suggesting that CED-4 probably could not form higher order complexes with CED-9 and its aptamers.

Some CED-4-binding Surfaces on CED-9 Are Important for Aptamer Binding—Our results indicate that R9-2 and R9-7 can form ternary complexes with CED-9/EGL-1 but not with CED-9/CED-4. To identify the CED-9 surface areas important for the binding of these two aptamers, we tested their binding to several CED-9 mutant proteins that contained amino acid substitutions in various surface exposed residues (11). As shown in Fig. 4*A*, the majority of CED-9 mutations that affect the binding between CED-9 and R9-2 also affect the binding between CED-9 and R9-7, confirming that these two aptamers share over lapping CED-9 binding sites. Because R9-2 and R9-7 have different secondary structures, they likely contact some distinct surface areas of

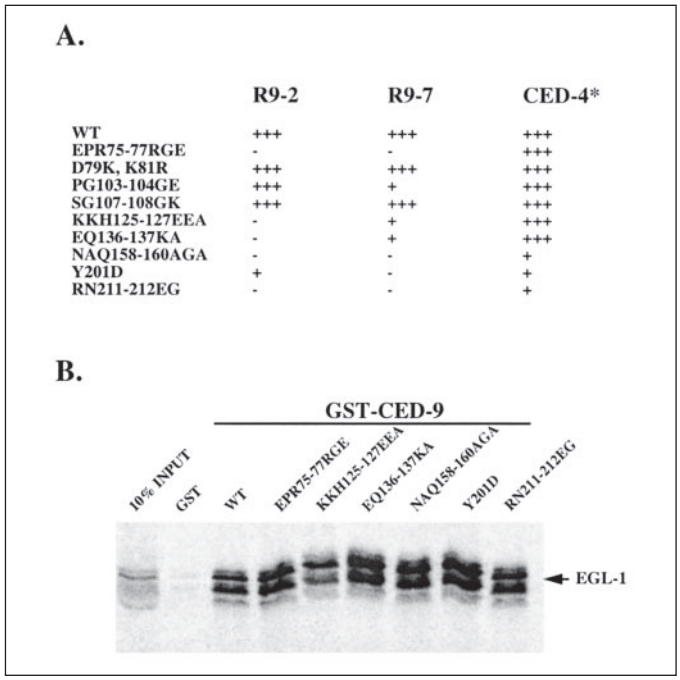

FIGURE 4. **CED-9 aptamers and CED-4 share overlapping CED-9 binding sites.** *A*, binding of R9-2 and R9-7 aptamers to CED-9 mutants. 2.5 μM wild-type or mutant GST-CED-9 proteins were incubated with ^{32}P-labeled aptamers and assayed with EMSA as describe in Fig. 1. "+++" indicates a strong aptamer/CED-9 binding based on the quantification of the gel shift bands with a PhosphorImager. "+" indicates a significantly reduced binding between an aptamer and a CED-9 mutant protein as compared with the binding of this aptamer to the wild-type CED-9 protein. "−" indicates no obvious binding between an aptamer and a CED-9 mutant protein. "*" indicates the interactions between CED-9 mutants and CED-4 as described by Yan *et al.* (11). *B*, interactions between CED-9 mutants and EGL-1. Equal amounts of wild-type or mutant GST-CED-9 proteins were incubated with [^{35}S]methionine-labeled EGL-1 and pulled down using glutathione-Sepharose beads as described previously (11) and viewed with a PhosphorImager. *WT*, wild type; *EPR75–77RGE*, E75R,P76G,R77E; *PG103–104GE*, P103G,G104E; *SG107–108GK*, S107G,G108K; *KKH125–127EEA*, K125E,K126E,H127A; *EQ136–137KA*, E136K,Q137A; *NAQ158–160AGA*, N158A,A159G,Q160A; *RN211–212EG*, R211E,N212G.

CED-9. Consistent with this hypothesis, CED-9(P103G,G104E), CED-9(K125E,K126E,H127A), CED-9(E136K,Q137A), and CED-9(Y201D) showed different binding affinities to these two aptamers (Fig. 4*A*). Importantly three sets of CED-9 mutations (N158A,A159G,Q160A; Y201D; and R211E,N212G) that disrupt binding of CED-9 to both aptamers are also critical for CED-4/CED-9 interaction (11).[4] Thus, these three CED-9 surface areas are important for both CED-4 and aptamer binding. Together with the EMSA results shown in Fig. 3*C*, these findings suggest that R9-2, R9-7, and CED-4 share overlapping CED-9 binding sites.

We further tested whether those CED-9 mutations that affected CED-9 binding to aptamers also affected CED-9/EGL-1 interaction. Using the glutathione *S*-transferase (GST) fusion protein pull-down assay, we found that wild-type and mutant GST-CED-9 proteins bound equally well to [^{35}S]methionine-labeled EGL-1 proteins, indicating that none of the CED-9 mutations that reduced or abolished aptamer binding to CED-9 significantly affected the interaction between CED-9 and EGL-1 (Fig. 4*B*). These results are consistent with the observations that EGL-1 and aptamers bind to different surface areas of CED-9 and provide further confirmation to the findings derived from the structural studies that EGL-1 and CED-4 bind to distinct surface areas of CED-9 (11, 12).

CED-9 Aptamers Induce Ectopic Cell Killing in C. elegans—Our *in vitro* studies indicated that two of the CED-9 aptamers, R9-2 and R9-7, bound to overlapping regions on CED-9 that appear to be different from the EGL-1 binding sites on CED-9. Furthermore our data indicated that these aptamers and CED-4 likely share some binding surfaces on CED-9, and the binding of aptamers to CED-9 may interfere with CED-4/CED-9 interaction. We thus tested whether these aptamers could promote cell killing *in vivo* by ectopically expressing CED-9 aptamers in touch receptor neurons under the control of the promoter of the *mec-7* gene (38).

TABLE 2
Overexpression of CED-9 aptamers induces ectopic cell killing in PLM touch receptor neurons

Transgene[a]	PLM survival				
	Low concentration (10 ng/μl)		High concentration (50 ng/μl)		
	Array[b]	*bzIs8*[c]	Array[b]	*bzIs8*[c]	*ced-3(n717); bzIs8*[c]
		%		%	%
None		100		100	100
P_{mec-7}R9-2	1	68	1	14	73
	2	74	2	23	89
P_{mec-7}R9-7	1	83	1	20	85
	2	85	2	19	75
Control aptamer	1	98	1	83	ND
	2	93	2	90	ND
P_{mec-7}*egl-1*			1	8	100
			2	7	97

[a] CED-9 aptamer expression constructs were injected into a *C. elegans* strain (*bzIs8*) together with a co-injection marker, pRF4 (50 μg/ml), which causes a Roller phenotype. P_{mec-7}*egl-1* construct (25 μg/ml) was injected with P_{ord-2}*rfp* (25 μg/ml), which directs red fluorescent protein expression in a few head neurons.
[b] Each numbered array represents an independent transgenic line. Thirty transgenic animals (animals with a Roller phenotype or red fluorescent protein expression in head neurons) from each line were scored for PLM survival (60 PLM neurons scored) using a fluorescent microscope. ND, not determined.
[c] *bzIs8* is an integrated transgene containing a P_{mec-4}*gfp* construct, which directs green fluorescent protein expression in six *C. elegans* touch receptor neurons and allows scoring of the PLM neurons.

We first generated transgenic lines with low copy extrachromosomal arrays expressing aptamer R9-2 or R9-7. In these transgenic animals, R9-2 caused ~30% killing of the PLM touch receptor neurons (68 and 74% PLM survival in two different transgenic lines) (Table 2). Similarly R9-7 caused ~15% of PLM killing (83 and 85% survival). In contrast, a control aptamer that did not bind CED-9 had very low PLM killing activity (93 and 98% PLM survival) (Table 2 and data not shown). The difference between R9-2 and R9-7 in cell killing is consistent with the finding that R9-2 had a higher binding affinity to CED-9 than did R9-7 (Fig. 2).

In *C. elegans*, the expression levels of a protein from transgenes normally correlate with the numbers of copy of a gene present in transgenes (39). To test whether these two aptamers, R9-2 and R9-7, can kill cells in a concentration-dependent manner, we generated high copy number transgenes expressing these two aptamers and found that they both displayed greatly enhanced killing activity in touch receptor neurons, resulting in ~80% killing of the PLM neurons (Table 2). The PLM killing activities of these two aptamers were close to that of EGL-1 (92% killing), which is a potent endogenous cell death inducer (Table 2). In contrast, the same control aptamer expressed in high copy transgene arrays had a marginal cell killing activity (Table 2). The low percentage of PLM killing caused by the control aptamer was probably due to the toxicity resulting from the expression of high concentrations of RNA in these neurons.

To determine whether R9-2 and R9-7 killed PLM neurons through the *C. elegans* programmed cell death pathway, we crossed the high copy transgenes expressing R9-2 or R9-7 into the *ced-3(n717)* mutant animals, which are defective in almost all programmed cell death. As shown in Table 2, PLM deaths induced by either R9-2 or R9-7 were

[4] N. Yan and Y. Shi, unpublished data.

significantly inhibited in *ced-3(n717)* animals, suggesting that the CED-3 caspase activity is important for the killing activities of these two aptamers. The residual killing observed in the *ced-3(n717)* mutant induced by CED-9 aptamers was comparable to the low level cell killing caused by the expression of the control aptamer, suggesting that the CED-3-independent death may be due to cell toxicity caused by high concentrations of RNA. However, we cannot rule out the possibility that CED-9 aptamers could cause some cell killing through a CED-3-independent mechanism (40). Nevertheless these results suggest that CED-9 aptamers induce ectopic PLM neuron deaths mainly through the CED-3 caspase and the *C. elegans* apoptotic program.

DISCUSSION

In an effort to isolate small molecular compounds that can modulate the activities of the Bcl-2 family proteins, we used the SELEX strategy to identify small RNA molecules that bind to the *C. elegans* Bcl-2 homologue CED-9 with high binding affinity and specificity. We isolated and characterized five RNA aptamers for CED-9 that can be categorized into three groups based on their secondary structures. Detailed biochemical analyses of two of these aptamers, R9-2 and R9-7, indicated that they can form ternary complexes with CED-9 and EGL-1, suggesting that these aptamers and EGL-1 bind to distinct surface areas on CED-9. In contrast, another proapoptotic protein, CED-4, could not form a ternary complex with CED-9 and its aptamers (Fig. 3*C*). Analyses of the interactions between R9-2 or R9-7 and several CED-9 mutants that are defective in binding to CED-4 revealed that these two aptamers and CED-4 share overlapping CED-9 binding sites (Fig. 4). Importantly overexpression of R9-2 or R9-7 induced robust ectopic cell killing that was largely dependent on the CED-3 caspase activity, suggesting that they are true small molecule cell death inducers. The cell killing effect of R9-2 or R9-7 is likely due to their interference with CED-9/CED-4 interaction *in vivo* that may antagonize the inhibitory activity of CED-9 on CED-4 and thus trigger apoptosis in those cells where the aptamers are expressed. The importance of the CED-3 caspase activity for the cell killing activity of CED-9 aptamers is consistent with this possibility and suggests that these aptamers act upstream of *ced-3* to induce cell killing.

Because *in vitro* selected RNA aptamers usually bind to specific domains of their target proteins with high affinity and specificity, they can often be used to probe the function of a specific protein domain or to discriminate the functions of highly homologous proteins. For example, an *in vitro* selected RNA aptamer was used to discriminate the roles of two highly homologous protein, cytohesin 1 and cytohesin 2, in regulating gene expression in response to serum stimulation (30). This aptamer binds to the N-terminal segment of cytohesin-2 specifically and can down-regulate expression of genes mediated though its serum response element and reduce mitogen-activated protein kinase activation in HeLa cells, suggesting a specific role of cytohesin-2 but not cytohesin-1 in serum-mediated transcriptional activation in nonimmune cells. In our study, two aptamers that we isolated specifically recognized potential CED-4 binding sites on CED-9 without interfering with the binding of EGL-1 to CED-9, providing further confirmation to the findings derived from the structural studies that CED-4 and EGL-1 bind to different surface pockets of CED-9 (11, 12). Thus these two aptamers and the other CED-9 aptamers yet to be characterized in detail can be very useful reagents for probing different or unknown functional domains of CED-9 in regulating programmed cell death in *C. elegans*. Our study further suggests that isolation of aptamers for other key cell death regulators in *C. elegans* will likely provide another powerful tool to facilitate the understanding of cell death activation in *C. elegans* and the characterization of poorly understood cell death regulators such as the proapoptotic protein CED-4. Compared with another RNA-based technique, RNA interference, which reduces or abolishes the activities of proteins of interest at the mRNA level, aptamers can achieve the same goal at the protein level but with higher resolution and specificity, especially for domain- or function-specific knock-out of proteins with multiple domains or functions.

Increasing evidence has shown that RNA aptamers are good lead compounds for developing diagnostic or therapeutic agents for treating human diseases (17). As a model experimental organism, *C. elegans* is being used to screen for compounds in drug development (41). The conservation of cell death pathways between nematodes and humans indicates that *C. elegans* can probably be used as an animal system to search for compounds that can modulate apoptosis *in vivo*. In both nematodes and humans, CED-9, Bcl-2, and Bcl-xL are the major cell death inhibitors and share similar protein structures (11, 42, 43). Furthermore Bcl-2 can partially substitute for the function of CED-9 in *C. elegans*, suggesting that they may interact with the same cell death regulators and share crucial functional domains (14, 44). Thus our successful isolation and characterization of potent CED-9 aptamers not only can provide important insights into how Bcl-2 proteins may interact with other cell death factors to control the activation of apoptosis but also suggest that a similar strategy can be applied to isolate aptamers for Bcl-2, Bcl-xL, and other Bcl-2 family proteins. Elevated expression of Bcl-2, Bcl-xL, and other Bcl-2 family proteins has been implicated in contributing to the development of a wide variety of human cancers and human diseases (10). Potent RNA aptamers specific for Bcl-2, Bcl-xL, or other Bcl-2 family proteins will be useful not only for studying how Bcl-2 family proteins regulate activation of apoptosis but also for developing new diagnostic reagents or therapeutic drugs to detect or treat those human diseases caused by abnormal apoptosis.

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