The *C. elegans* B-cell Lymphoma 2 (*bcl-2*) Homolog Cell Death Abnormal 9 (*ced-9*) Associates with and Remodels Lipid Membranes

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Abstract: Bcl-2 proteins associate with and remodel mitochondria to regulate apoptosis. While the *C. elegans* Bcl-2 homolog CED-9 constitutively associates with mitochondria, it is unclear whether or not this association reflects an innate ability of CED-9 to directly remodel mitochondrial membranes. To address this question, we have characterized the effects of recombinantly expressed and purified CED-9 on synthetic lipid vesicles. We found that CED-9 associates with anionic lipid vesicles at neutral pH, and that association can occur independently of the C-terminal transmembrane domain. Membrane association changes the environment of CED-9 tryptophans and results in an apparent increase in α -helical structure. Upon association, CED-9 alters the permeability of membranes resulting in leakage of encapsulated dyes. Furthermore, this membrane remodeling promotes membrane fusion upon protonation of CED-9. Bypass of this protonation trigger can be achieved by mutating two conserved glutamates (E187K/E190K) or removing the N-terminal 67 residues. Together, these *in vitro* results suggest that CED-9 retains the amphitropic ability of mammalian Bcl-2 proteins to associate with cellular membranes. We therefore discuss the possibility that CED-9 and other Bcl-2 homologs localize at mitochondria to regulate mitochondrial homeostasis by either modulating mitochondrial membrane permeability or fusion.

Keywords: Bcl-2 proteins; *C. elegans*; protein amphitropism; mitochondrial apoptosis; mitochondrial morphology; membrane fusion; fusion protein; phospholipid vesicle

INTRODUCTION

Mitochondria contain many proteins, such as cytochrome c, endonuclease G and AIF-1 that trigger apoptosis when released into the cytosol.¹ This trigger is tightly controlled by the Bcl-2 family of proteins, which promotes or inhibits apoptosis by disrupting or maintaining mitochondrial membrane integrity.^{2,3} This regulation of mitochondrial membrane integrity has been proposed to involve the amphitropic nature of Bcl-2

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proteins that allows reversible association with cell membranes.^{4–7} Studying this amphitropism and its consequences on function in mammalian systems has been difficult, however, due to the presence of multiple homologs with either redundant or opposite functions. For instance, a defect in the structure of the overall mitochondrial network is only apparent after disrupting two Bcl-2 homologs, Bax and Bak.³

C. elegans presents a tractable system to study how Bcl-2 amphitropism regulates mitochondrial membrane integrity as CED-9 is the only known multidomain Bcl-2 homolog. While only one homolog exists, CED-9 is capable of both promoting and inhibiting apoptosis depending on the particular cell type.³ This dual function of CED-9 in apoptosis recapitulates the functions found in the more numerous mammalian Bcl-2 family, and suggests that *C. elegans* may provide a good system to study the importance of Bcl-2 amphitropism and membrane remodeling activities. Further supporting this system is the observation that CED-9 constitutively localizes to mitochondrial outer membranes.^{8–10} Although cytochrome c release does not appear to be required to activate apoptosis in *C. elegans*,¹¹ mitochondrial localization of CED-9 appears essential for full apoptotic activity¹⁰ and the release of the nematode homologs of endonuclease G and AIF-1 do potentiate apoptosis.³ The mitochondrial localization of CED-9 and the conserved role of *C. elegans* mitochondrial proteins in apoptosis suggest that CED-9 may regulate their release through direct association and remodeling of membranes.

Evidence for direct remodeling of mitochondrial membranes by Bcl-2 proteins comes primarily from studies with the human Bcl-2 homolog Bax. When activated by apoptotic stimuli, Bax associates with mitochondrial outer membranes and increases membrane permeability resulting in cytochrome c release. This increased permeability is proposed to involve a homo-oligomeric complex of membrane-embedded Bax that forms membrane pores.¹² To counteract the Bax activity and stabilize membrane integrity, antiapoptotic Bcl-2 homologs are thought to also embed within membranes. In this case, antiapoptotic homologs like Bcl-x_L are proposed to bind membrane embedded Bax in a “dominant negative” fashion, thereby preventing formation of functional pores.² It remains unclear, however, whether membrane association and remodeling by antiapoptotic Bcl-2 proteins^{13,14} directly contribute to cell survival rather than solely inhibiting pro-apoptotic proteins.

To study whether membrane remodeling by Bcl-2 proteins contributes to apoptosis in *C. elegans*, it is reasonable to first determine whether or not CED-9 possesses the amphitropic ability to associate with membranes. Three pieces of evidence suggested that this might be the case. First, CED-9 contains the core Bcl-2 domain that has been shown to be amphi-

tropic.^{12–14} In CED-9 this domain lies between a nonconserved N-terminal extension and a C-terminal transmembrane domain.¹¹ Second, increased CED-9 expression in both *C. elegans* and mammalian cells results in excessively fused mitochondrial membranes, possibly the result of membrane remodeling.^{9,15,16} Finally, a recent report found that CED-9 lacking the C-terminal transmembrane domain preserves mitochondrial membrane integrity in isolated rat mitochondria during apoptotic stresses by dismantling ceramide channels.¹⁷ Combined, these data suggest that CED-9 may be able to interact with membranes, and this amphitropism may promote membrane remodeling. Here, we present the first detailed analysis of CED-9-membrane interactions using purified recombinant protein and lipid vesicles.

RESULTS

For several human Bcl-2 homologs, the core Bcl-2 domain exhibits amphitropism and will associate with membranes even after removal of the C-terminal transmembrane domain.^{13,14,18,19} To directly test whether or not CED-9 also exhibits amphitropism via only the core Bcl-2 domain, we used a combination of synthetic lipid vesicles and purified recombinant CED-9 lacking the C-terminal 29 residues. This experimental system provides several advantages when compared to other systems to study protein-membrane interactions: (1) any membrane association must be direct and not bridged via protein receptors on the membrane as CED-9 is the only protein present in this system, (2) spectroscopic measurements report directly on the structure of CED-9, (3) the creation of lipid vesicles in aqueous solutions allow for various experimental designs such as fractionation of intact vesicles,²⁰ encapsulation of small molecules,²¹ and incorporation of labeled lipids,²² and (4) multiple methods exist to assay the morphology of vesicles.²³

CED-9 displays amphitropism and associates with membranes independently of the C-terminal transmembrane domain

To assay whether or not CED-9 exhibits amphitropism, we tested whether or not protein lacking the C-terminal transmembrane domain could directly interact with membranes by performing co-sedimentation studies using purified protein and lipid vesicles.^{14,20} These lipid vesicles contained a 60:40 molar ratio of phosphatidylcholine:phosphatidylglycerol headgroups that provide a negatively charged membrane surface previously shown to be required for efficient Bcl-2 membrane association.^{14,24} After incubation of CED-9 (5 μ M protein) with large unilamellar vesicles (2 mM lipid) for 2 hours at 23°C, we sedimented samples by centrifugation. Aliquots from the supernatant and pellet were subsequently

Figure 1. CED-9 associates with membranes independently of the C-terminal transmembrane domain. Five micromolar of recombinant CED-9 protein lacking the C-terminal transmembrane domain was incubated with or without anionic lipid vesicles (2 mM total lipid, 1:400 protein: lipid ratio). After incubation for 2 hours at 23°C, samples were sedimented and fractionated as described in Materials and Methods. (a) At neutral pH, ~66% ($\pm 0.8\%$ s.e.m.) of CED-9 associated with membranes in contrast to a control protein (maltose binding protein, MBP) that did not show any enrichment in the pellet fraction in the presence of membranes. More CED-9 associated with membranes after acidification of the buffer, reaching ~91% ($\pm 0.7\%$ s.e.m.) at pH 5.0. At this condition, however, CED-9 had decreased solubility (see Materials and Methods). S, supernatant; P, pellet. (b) Membrane association was examined as a function of increasing concentration of NaCl at pH 7.0. In the absence of NaCl, ~74% of CED-9 associated with membranes. We note that 13.5 mM KCl was carried over from the protein stock solution. Error bars represent the standard error ($n = 3$).

analyzed by either SDS-PAGE [Fig. 1(a)] or Bradford assay (data not shown) to quantitate protein concentration in each fraction. Regardless of the detection method, 66% ($\pm 0.8\%$; s.e.m.) of CED-9 at neutral pH was found in the pellet fraction [Fig. 1(a)]. This finding is in contrast to a similar human Bcl-x_L construct that does not associate with membranes at neutral pH.¹⁴ CED-9 sedimentation required membranes, as in the absence of membranes less than 5% of total protein was found in the pellet fraction. This membrane association was specific to CED-9, as neither human Bcl-x_L nor a control protein (maltose binding protein, MBP) showed any enrichment in the pellet fraction in the presence of membranes. These results indicate that CED-9 lacking the C-terminal transmembrane domain can directly associate with membranes independently of any protein receptor.

As human Bcl-x_L has previously been shown to involve electrostatic interactions during membrane association,^{14,24} we investigated this by measuring CED-9 membrane association using the co-sedimentation assay as a function of increasing salt concentration. As seen in Figure 1(b), membrane association steadily decreased from 74% to 11% between 13.5 and 1000 mM salt. Notably, at a physiological salt concentration of 150 mM, ~28% of CED-9 associated with membranes as measured by this assay. These results suggest that like human Bcl-x_L, CED-9 membrane association involves a significant electrostatic component.

Consistent with an electrostatic component to amphitropism, the membrane affinity of several human Bcl-2 homologs increases upon protonation.^{14,18} We therefore tested whether or not protonation similarly affected the membrane affinity of CED-9. When the buffer was acidified to pH 6.0, CED-9 membrane association increased to 89% ($\pm 1.3\%$) [Fig. 1(a)]. As the pK_a of the phospholipid phosphate moieties are below pH 3.5,²⁵ the increased membrane affinity upon acidification of the buffer is likely due to protonation of CED-9. Further acidification of the buffer to pH 5.0 resulted in 91% ($\pm 0.7\%$) of CED-9 in the pellet fraction. We note, however, that CED-9 solubility decreased at pH 5.0, complicating measurements of membrane association under this condition [see Fig. 1(a) and Materials and Methods].

To examine the effects of membrane association on CED-9, we first assessed secondary structure by far-UV circular dichroism. In the absence of membranes at neutral pH, CED-9 lacking the C-terminal domain had a CD spectrum with a maximum near 192 nm and minima at 208 and 222 nm suggesting an α -helical content of ~38% (Fig. 2) according to the method of Luo and Baldwin.²⁶ This secondary structure is consistent with crystallographic structures of the core Bcl-2 domain of CED-9 that shows a bundle of α -helices.¹¹ When CED-9 was incubated with membranes, all maxima and minima increased, suggesting an increase in helical content to ~44%. Similar results were obtained with CED-9 at pH 6.0 and pH 5.0, indicating that increased membrane association did not result simply from protein unfolding (data not shown). Together, these data indicate that upon membrane association CED-9 retains and possibly increases in α -helical structure.

A change in protein structure was further suggested by monitoring tryptophan fluorescence emission. In the absence of membranes at neutral pH, CED-9 lacking the C-terminal domain emitted a tryptophan emission peak at 334 nm [Fig. 3(a)]. Upon incubation with membranes, the intensity of this peak doubled. Such changes in tryptophan fluorescence are consistent with either an altered protein structure or an altered aqueous/membrane

Figure 2. CED-9 retains secondary structure after association with membranes. CED-9 protein (2.5 μ M) lacking the C-terminal transmembrane domain was incubated with or without lipid vesicles at pH 7.0 for 1 hour at 23°C (250 μ M total lipid, 1:100 protein:lipid ratio). Far-UV CD spectra with minima at 208 and 222 nm indicated that CED-9 possessed α -helical secondary structure both in solution (○, estimated 38% according to Ref. 26) and after association with membranes (●, estimated 44%).

environment. Although differentiating between these two possibilities is difficult, due in part to the presence of multiple tryptophan residues (see Materials and Methods), we nonetheless took advantage of this signal change to measure the membrane affinity of CED-9 lacking the C-terminal domain by measuring a partition coefficient (K_x).²⁰ By monitoring the fluorescence intensity of CED-9 at 335 nm as a function of increasing lipid concentration, we observed saturation of CED-9 signal that could be fitted to obtain a partition coefficient of $K_x = 1.4 \pm 0.1 \times 10^6$ [Fig. 3(b)]. Similar experiments at pH 6.5 resulted in a best fit value of $K_x = 1.3 \pm 0.1 \times 10^7$ [Fig. 3(c)], suggesting that more CED-9 associates with membranes after protonation. These estimated amounts of CED-9 associating with membranes are greater than the amounts measured from the co-sedimentation studies described earlier (Fig. 1). This discrepancy may result from differences in protein concentration (5 μ M vs 1 μ M), acyl-chain composition (see Materials and Methods), or sensitivity to weak interactions. Regardless, the observed membrane association and the trend as a function of pH remain reproducible between experiments. Therefore, we interpret these data to indicate a specific interaction between CED-9 and anionic membranes.

To examine how the C-terminal transmembrane domain of CED-9 contributes to membrane association, we purified full-length CED-9. Though yield and purity were low (see Materials and Methods), we were able to use tryptophan emission fluorescence to measure membrane association. Similar to CED-9 lacking the C-terminal transmembrane domain, full-length CED-9 at neutral pH emitted a tryptophan emission peak at 335 nm in the absence

of membranes (Supporting Information Figure 1a). Incubation with membranes resulted in a 1.7-fold increase in peak intensity. Titrating lipids and measuring fluorescence intensity at 336 nm resulted in a best fit value for membrane partitioning of $K_x = 2.0 \pm 0.2 \times 10^6$ (Supporting Information Figure 1b), a value similar to that of CED-9 lacking the C-terminal transmembrane domain. These data indicate that under the conditions assayed similar amounts

Figure 3. Membrane association alters the environment of CED-9 tryptophans. (a) Tryptophan emission spectra (ex. 295 nm) at neutral pH for 1 μ M CED-9 lacking the C-terminal transmembrane domain exhibited an increase in fluorescence intensity in the presence of lipid vesicles (200 μ M total lipid, 1:200 protein:lipid ratio) (●) when compared to protein alone (○). (b,c) The change in fluorescence intensity at 335 nm was monitored as a function of lipid concentration at pH 7.0 to calculate a partition coefficient of $K_x = 1.3 \pm 0.1 \times 10^6$. (c) A similar titration at pH 6.5 resulted in a calculated partition coefficient of $K_x = 1.4 \pm 0.1 \times 10^7$.

of CED-9 associate with membranes regardless of whether or not the C-terminal transmembrane domain is present. These findings suggest that the presence of a C-terminal transmembrane domain does not substantially alter the mode of CED-9 membrane association in this reconstituted system. We finally tested whether or not the C-terminal transmembrane domain bypassed the requirement of negatively charged membrane surfaces for CED-9 to associate with membranes. When we added neutral membranes to full-length CED-9, we did not observe any change in fluorescence signal (Supporting Information Figure 1c). Although this result does not preclude an interaction that does not alter tryptophan fluorescence signals, this lack of a change in signal suggests that electrostatic interactions are necessary for full-length CED-9 to associate with membranes.

Membrane association results in permeabilization and lipid exchange

To investigate the consequences of CED-9 association on membranes, we assayed whether or not CED-9 altered membrane permeability. Specifically, we monitored membrane permeability by creating vesicles encapsulating the ANTS/DPX fluorophore-quencher dye pair. If membrane permeability increases such that either dye is released into the buffer solution, ANTS fluorescence will increase due to a decrease in local DPX quencher concentration. Such permeability assays are sensitive to changes in membrane structure such as membrane dissolution,²⁷ pore or channel formation,²⁸ or “leaky” membrane fusion.²⁹

Incubation of dye encapsulated vesicles (100 μ M lipid) with 500 nM CED-9 at neutral pH resulted in a rapid increase of ANTS fluorescence (data not shown). This is in contrast to vesicles incubated without CED-9, which did not show any increase in ANTS fluorescence, indicating that membrane integrity normally remained intact throughout the assay. These results indicate that CED-9 increases membrane permeability upon association. To further characterize the effect of CED-9 on membrane integrity, we measured dye release as a function of CED-9 concentration and found a binding isotherm that is typical of noncooperative binding [Fig. 4(a)]. We observed dye release at concentrations as low as 25 nM, which corresponds to roughly 18 CED-9 molecules per vesicle assuming 10^5 phospholipids per vesicle and a partition coefficient of $K_x = 1.4 \pm 0.1 \times 10^6$.

As Bcl-2 proteins have been observed to play a role in mitochondrial membrane fusion,³ we tested whether or not dye release resulted from imperfect “leaky” membrane fusion between lipid vesicles. To accomplish this, we monitored the exchange of phospholipids between differentially labeled vesicle populations.²² Specifically, one population of vesicles con-

Figure 4. Membrane structure is altered upon CED-9 association. (a) Vesicles encapsulating the fluorophore-quencher pair ANTS: DPX (100 μ M total lipid) were incubated with various amounts of CED-9 at neutral pH. Increased ANTS fluorescence resulted from a dilution of the DPX quencher, indicating a change in membrane permeability. Increasing concentrations of CED-9 resulted in increasing amounts of dye leaked from vesicles. Samples were incubated at room temperature for 2 hours. Error bars represent the standard deviation from three experiments (except 300 nM where $n = 2$). (b) Vesicles composed of phospholipids covalently labeled with the NBD/rhodamine FRET pair were incubated with CED-9 at neutral pH with a ninefold excess of unlabeled vesicles (100 μ M total lipid). Increased NBD fluorescence resulted from a dilution of rhodamine labeled phospholipids with lipids from unlabeled vesicles, indicating a mixing of lipids between the two vesicle populations. Increasing concentrations of CED-9 resulted in increasing amounts of lipid mixing between vesicle populations. Samples were incubated for 3 hours at room temperature. Error bars represent the standard deviation from three experiments.

tained both phospholipids covalently labeled with the fluorophore NBD and quenching concentrations of phospholipids covalently labeled with rhodamine. These vesicles were then mixed with an excess of unlabeled vesicles. If membrane fusion occurred between vesicles, lipids would mix between vesicles and NBD fluorescence would increase due to a decrease in the rhodamine quencher concentration.

Upon incubation of 500 nM CED-9 at neutral pH with labeled vesicles and ninefold excess of unlabeled vesicles (100 μ M total lipids), an increase in

Figure 5. CED-9 promotes membrane fusion under acidic buffer conditions. Vesicles (200 μ M total lipid, 1:200 protein: lipid ratio) with or without 1 μ M CED-9 were incubated at various pH conditions for 2 hours and examined by both dynamic light scattering and negative stain EM. (a) Vesicles alone were stable at all pH conditions examined, showing a monodisperse sample with a z-average diameter of 113 nm, 112 nm, and 112 nm at pH 7.0, 6.0, and 5.0, respectively. In contrast, vesicles incubated with CED-9 displayed a second larger population at acidic buffer conditions. (b) These larger vesicles were apparent when visualized directly using negative stain EM.

NBD fluorescence occurred $\sim$ 3 minutes after protein addition (Supporting Information Figure 2). This is in contrast to incubation of vesicles without CED-9, which showed no apparent increase in NBD fluorescence, indicating that phospholipids normally do not exchange between vesicles during the assay. These results indicate that CED-9 promotes phospholipid exchange between two distinct vesicle populations shortly after membrane association. This result was further characterized by measuring phospholipid exchange as a function of CED-9 concentration [Fig. 4(b)]. Once again, we observed activity at concentrations as low as 25 nM consistent with the dose response of CED-9 on membrane permeability. Interestingly, the binding isotherm for lipid mixing suggests a cooperative reaction in contrast to the non-cooperative binding isotherm for dye release [Fig. 4(a)]. Such differences in cooperativity are consistent with mechanisms whereby dye release results from a single membrane partitioning event, while lipid mixing results from the interaction of at least two membrane associated molecules. Thus, the apparent cooperative behavior in lipid mixing experiments

suggests that more than one CED-9 molecule is required to promote phospholipid exchange. This behavior also suggests that phospholipid exchange is not simply the result of membrane destabilization.

CED-9 amphitropism promotes membrane fusion after protonation

Phospholipid exchange between distinct vesicle populations can occur through either the formation of hemifusion intermediates or the complete fusion of two membranes.³⁰ The former event results in fusion of only outer bilayer leaflets, while the latter event results in fusion of the entire bilayer. Under the assumption that complete fusion produces vesicles with a larger diameter than the starting population, we characterized the morphology of lipid vesicles upon CED-9 association using dynamic light scattering and negative stain electron microscopy.

Vesicles alone at neutral pH had a monodisperse size distribution with a z-average diameter of 113 nm, consistent with vesicles observed by electron microscopy (Fig. 5). When vesicles were incubated with CED-9 at neutral pH, vesicles retained a similar

Figure 6. Two different mutations allow CED-9 to promote membrane fusion under neutral buffer conditions. Two CED-9 mutants were separately examined for membrane association and membrane fusion activity: one which reversed the charge of two glutamates on a putative membrane binding surface (E187K/E190K) and one which removed an N-terminal domain that is not conserved among mammalian Bcl-2 homologs (ΔN). (a) At all pH conditions examined, the amount of mutant CED-9 protein that associated with membranes was similar to wild-type protein. (b,c) A second larger vesicle population was present when either mutant protein was included in the reactions at all pH conditions examined, including neutral buffer conditions.

unimodal distribution with a z -average diameter of 115 nm, again similar to that observed by electron microscopy (Fig. 5). Thus, even though CED-9 associates with and remodels membranes at neutral pH, it does not promote complete membrane fusion at neutral pH.

We next tested whether or not protonation allowed CED-9 to promote complete membrane fusion. Such activity regulation exists for human Bcl-x_L, where protonation both increases membrane affinity and activates membrane remodeling activity.^{7,13} Incubating vesicles with CED-9 at decreased pH resulted in larger vesicles (Fig. 5). The z -average diameter of vesicles increased from 115 nm at pH 7.0 to 138 nm at pH 6.0, and 190 nm at pH 5.0. Solely decreasing the pH to 6.0 or 5.0 did not alter vesicle morphology by either dynamic light scattering or electron microscopy.

Since these samples were not monodisperse, we further analyzed these data to resolve different sized species. As shown in Figure 5(a), a second larger population was observed when vesicles were incubated with CED-9 under acidic conditions. These larger vesicles could be readily observed by electron microscopy [Fig. 5(b)]. These results suggest that CED-9 protonation both increases membrane affinity and allows CED-9 to promote membrane fusion.

Two different mutations allow CED-9 to promote membrane fusion at neutral pH

Protonation of Bcl-2 proteins *in vivo* might occur during apoptosis when cytosol is transiently acidified.³¹ As this model remains debated,³² we sought to identify mutations that would allow CED-9 to promote membrane fusion at neutral pH. One potential regulatory region is glutamate 187 and 190, a pair of acidic amino acids conserved throughout most Bcl-2 homologs.⁷ These glutamates are located at the tip of a putative membrane insertion domain and have been proposed to regulate interactions with negatively charged membranes. In support of this idea, mutation of residues at similar positions in human Bcl-x_L and the structurally similar *Diphtheria* toxin translocation domain alters both membrane affinity and membrane remodeling activity.^{7,33}

We first created a CED-9(E187K/E190K) construct where both glutamates were mutated to lysines. A similar construct retains the ability to both regulate apoptosis and promote mitochondrial fusion *in vivo*¹⁵ (unpublished observations). *In vitro*, reversal of these charges from acidic to basic did not significantly affect membrane association at neutral pH by vesicle sedimentation assays [Fig. 6(a)]. Mutation of these two residues did, however, dramatically increase membrane fusion activity at neutral pH.

When vesicles were incubated with CED-9(E187K/E190K) for 2 hours, a *z*-average diameter of 139 nm was observed, a size comparable to the *z*-average diameter of 138 nm observed when wild-type CED-9 was similarly incubated with vesicles at pH 6.0 [Figs. 5(a) and 6(b)]. This larger distribution of vesicles was confirmed by negative stain electron microscopy [Fig. 6(c)]. Additionally, protonation of CED-9(E187K/E190K) further increased the *z*-average diameter of vesicles to 166 nm at pH 6.0 and 216 nm at pH 5.0.

A second potential regulatory region of CED-9 is the N-terminal extension that contains two caspase cleavage sites.³⁴ Cleavage at these caspase sites in CED-9 and human Bcl-2 homologs results in several altered membrane activities.^{24,35,36} We therefore created a Δ NCED-9 construct lacking the N-terminal 67 residues. A similar construct retains the ability to both regulate apoptosis and promote mitochondrial fusion *in vivo*.^{10,15} *In vitro*, removal of the N-terminal extension did not significantly affect membrane association at neutral pH by vesicle sedimentation assays [Fig. 6(a)]. This truncation did, however, dramatically increase membrane fusion activity at neutral pH. Similar to CED-9(E187K/E190K), incubation of vesicles with Δ NCED-9 resulted in a larger *z*-average diameter of 163 nm. The size distribution was more heterogeneous than for wild-type CED-9 or CED-9(E187K/E190K), as reflected in both the multimodal distribution [Fig. 6(b)] and large vesicles observed by negative stain electron microscopy [Fig. 6(c)]. Protonation of Δ NCED-9 at pH 6.0 did not significantly change the *z*-average diameter of vesicles (166 nm), but did lead to an increase at pH 5.0 (191 nm). Taken together, these data suggest that the core Bcl-2 domain of CED-9 is sufficient to remodel membranes, and that this activity is regulated by the N-terminal extension.

DISCUSSION

In this study, we show *in vitro* that CED-9 can associate with membranes via a mechanism that is independent of a C-terminal transmembrane domain and likely similar to the amphitropic class of proteins. This mechanism suggests that *in vivo* a second means of mitochondrial membrane association may be possible in addition to the previously described C-terminal transmembrane domain.^{9,10} This dual membrane targeting mechanism is reminiscent of mammalian Bcl-2 homologs^{14,18,19} and suggests that CED-9 may share many of the properties of mammalian Bcl-2 proteins. In fact, like mammalian Bcl-2 proteins,^{12–14,18} we find that CED-9-membrane association results in extensive membrane remodeling and is consistent with a model whereby Bcl-2 proteins directly modulate membrane structure during apoptosis and possibly normal mitochondrial homeo-

stasis. For CED-9, such a role would cast it as an active participant at mitochondria and not simply a passive tether sequestering pro-apoptotic factors.³ This role would explain why CED-9 retains the mitochondrial localization characteristic of mammalian homologs, and supports models where direct mitochondrial interactions by CED-9 and mammalian Bcl-2 homologs regulate apoptosis and mitochondrial homeostasis.^{9,10,15,16,37}

CED-9 adopts multiple conformations at mitochondrial membranes

Our data extend previous studies by suggesting that CED-9 might adopt at least two distinct conformations at mitochondrial membranes. One conformation exists as a “tail anchored protein” where only the C-terminal transmembrane inserts into mitochondrial membranes. This conformation is supported by the finding that the C-terminal transmembrane domain of CED-9 is sufficient to target GFP to mitochondrial outer membranes.¹⁰ Such a conformation allows the C-terminal tail to interact with and regulate mitochondrial apoptotic proteins such as ceBNIP3 and WAN-1.^{38,39} This conformation allows the core Bcl-2 domain to remain solvent accessible and available to interact with cytosolic apoptotic proteins such as CED-4.^{8,11}

Our data support a model in which a second conformation might exist where multiple portions of CED-9 associate with mitochondrial membranes. This conformation is supported by the finding that CED-9 lacking the C-terminal transmembrane domain associates with membranes (this study). Such a conformation would allow CED-9 to remodel membranes, potentially to modulate membrane integrity (discussed later). This ability of CED-9 to associate with membranes independently of the C-terminal transmembrane domain raises the interesting possibility that *in vivo*, full-length CED-9 may sit on mitochondria with the C-terminal transmembrane domain tucked into the BH3 binding pocket. Although direct evidence remains to be discovered that CED-9 adopts such a conformation, this conformation has been observed for human Bax and Bcl-w.^{40,41} Such a conformation would allow for regulated access to both the CED-9 BH3 binding pocket and C-terminal transmembrane domain.

Given our findings that CED-9 can associate with membranes independently of the C-terminal transmembrane domain *in vitro*, it is curious that earlier *in vivo* localization studies did not observe mitochondrial enrichment with similar constructs.^{9,10} One possibility is that the ionic strength inside cells limits the amount of CED-9 that associates with membranes in the absence of a C-terminal transmembrane domain [Fig. 1(b)]. Additionally, the concentration of protein and membrane in cells may result in only a small fraction of membrane

association. Because diffuse GFP signals in the cytosol juxtapose GFP signals on mitochondrial membranes, it would be difficult to detect such a population. Careful fractionation studies may reveal such an association. Finally, the core Bcl-2 domain of CED-9 may only associate with membranes under specific conditions due to direct regulation by other proteins or indirect regulation of membrane structure. As previous studies primarily examined localization in healthy cells, it might be fruitful to study localization in cells undergoing apoptotic stress. Such differential localization would be consistent with human Bax.¹⁹

Possible roles for CED-9 membrane remodeling

The membrane remodeling activity of CED-9 observed *in vitro* may be involved in two nonmutually exclusive processes. The first process may be to release mitochondrial pro-apoptotic molecules such as CPS-6 and WAH-1. Release of these molecules by CED-9 would be consistent with the pro-apoptotic function of CED-9.³ To determine whether CED-9 could release these molecules, it will be necessary to characterize what size proteins pass through CED-9 permeabilized membranes.^{12,24} Additionally, it would be interesting to see whether or not CPS-6 and WAH-1 are still released from mitochondria in cells where CED-9 is targeted to a cytosolic membrane distinct from mitochondria such as endoplasmic reticulum.¹⁰ If CED-9 releases these proteins by directly remodeling mitochondrial membranes, then sequestering CED-9 away from mitochondria should prevent their release.

A second process that may involve CED-9 membrane remodeling is mitochondrial fusion. This process normally involves fusion of outer membranes by mitofusins located on two separate mitochondria, followed by fusion of inner membranes.⁴² It has recently been proposed that CED-9 promotes these two events primarily by regulating mitofusin activity.¹⁶ This model is based on the finding that increased CED-9 expression in cells lacking the mitofusin FZO-1 contain clustered rather than fused mitochondria. However, as this experiment examined inner membrane markers, it is unclear whether outer membranes were still fused in these cells. Such a finding would imply that CED-9 membrane remodeling plays a direct role in mitochondrial outer membrane fusion. Consistent with this idea, electron microscopy analysis has revealed that increased CED-9 expression can produce "hyperfused" outer membranes.^{15,16} Interestingly, the structurally similar *Diphtheria* toxin translocation domain has been reported to promote fusion of early endosomes.⁴³

One final question is how membrane remodeling activity may be regulated. We present evidence that CED-9 has increased membrane fusion activity *in vitro* upon removal of the N-terminal extension (Fig.

6). This activity is consistent with the finding that a similar N-terminal truncated construct is fully competent at both regulating apoptosis and promoting mitochondrial fusion *in vivo*.^{10,15} This CED-9 region can in fact be cleaved off by the CED-3 caspase,³⁴ and similar cleaved fragments have been found in mammalian cells with activated caspases.³⁵ These findings raise the possibility that CED-9 membrane activity may be linked to caspase activation. Intriguingly, it has been observed that while full-length CED-9 localizes to mitochondrial outer membranes in mammalian cell lines, N-terminal truncations of CED-9 localize to mitochondrial inner membranes.³⁶ Understanding this differential localization may shed light into the role of CED-9 at membranes.

MATERIALS AND METHODS

Protein expression and purification

The plasmid pFT30.31 encodes CED-9 lacking the C-terminal transmembrane domain fused to pHis-Gβ1 and was previously described.¹⁵ Additional constructs were cloned using standard protocols, and all coding regions amplified by PCR were verified by sequencing. Plasmid pFT29.20 encodes wild-type CED-9 (residues 1–280), pFT47.8 encodes CED-9 lacking both the N-terminal extension and the C-terminal transmembrane domain (residues 68–251), and pFT49.83 encodes CED-9 lacking the C-terminal transmembrane domain with both glutamate 187 and glutamate 190 mutated to lysine.

Recombinant CED-9 was bacterially expressed and purified as a pHis-Gβ1 fusion construct as previously described.¹⁵ Briefly, Rosetta cells (Novagen) carrying the appropriate plasmid were grown in LB media, protein expression was induced using IPTG at 18°C, and fusion protein was purified from cell lysate using a Ni²⁺ chelating column. The fusion tag was cleaved off using 6xHis-TEV protease, leaving an N-terminal three amino acid cloning artifact (Gly-Glu-Phe). CED-9 was separated from both the fusion tag and TEV protease by a second pass over a Ni²⁺ chelating column, and a final pass over a Superdex 75 gel filtration column (GE Healthcare). The final protein was dialyzed into buffer containing 20 mM Tris pH 8.0, 250 mM KCl, 1 mM EDTA, and 1 mM DTT; concentrated to ~100 μM protein concentration; and stored at 4°C until use. Due to the presence of KCl in the storage buffer, all experiments contained at most 2.5–13.5 mM KCl, which did not significantly affect membrane association [Fig. 1(b)]. At no time were detergents used during purification.

All CED-9 constructs lacking the C-terminal transmembrane domain produced yields ~10 mg/L of LB media, and were judged to be >95% pure by Coomassie-stained gel electrophoresis. Full-length CED-9 expressed similar amounts of protein,

however, the majority of the protein was insoluble and produced yields less than 0.1 mg/L; purity ranged between 60 and 80%.

Maltose binding protein (MBP) was expressed as a fusion protein as previously described.⁴⁴ After cleavage and separation on a Superdex 75 column, the fractions containing MBP were retained for use in vesicle sedimentation assays.

Preparation of large unilamellar vesicles

All synthetic lipids were obtained from Avanti Polar Lipids (Alabaster, AL). The lipids were mixed in chloroform to the desired ratios, dried under a stream of nitrogen gas, and held under high vacuum for 2 hours. Unless otherwise indicated, lipids were generally resuspended at 10 mg/mL in distilled water, subjected to three freeze-thaw cycles, extruded eleven times through a 100 nm polycarbonate filter, and stored at 4°C until use.⁴⁵ 1,2-dioleoyl acyl chain phospholipids were generally used to allow comparison with previous studies.^{12,14,24,46–48}

Vesicle sedimentation assay

Lipid vesicles were created with 1,2-dibromostearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) at a 60:40 ratio, with 0.25% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (DOPE) to aid in visual detection of the vesicles. Vesicle sedimentation assays were performed with 5 μ M protein and 2 mM lipid (1:400 ratio), in a 100 μ L volume buffered with 20 mM sodium acetate and 20 mM potassium phosphate. Reactions were incubated with rotation for 2 hours at 23°C, and then subjected to centrifugation at increasing speeds (4000, 8000, and 14,000 rcf) for 30 minutes each.²⁰ The top 80 μ L was carefully drawn off and diluted into 4 $\times$ SDS loading buffer; the remaining 20 μ L which contained a mixture of protein and trace lipids was discarded. The pellet which contained the majority of lipids was resuspended in 100 μ L of buffer, and then diluted into 4 $\times$ SDS loading buffer. Fifteen microliter of each fraction was analyzed by SDS-PAGE and stained with Coomassie G-250.

We note that CED-9 lacking the C-terminal transmembrane domain was less soluble at pH 5.0. As shown in Figure 1, less protein was recovered in the supernatant from samples lacking vesicles. Approximately 70% of total protein was present at the bottom of centrifuge tubes and only recovered after resuspension using heated loading buffer (data not shown).

Circular dichroism spectropolarimetry

Lipid vesicles were created with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and DOPG at a 60:40 ratio. Far-UV spectra were collected for 2.5 μ M pro-

tein and 250 μ M lipid (1:100 ratio) in 50 mM potassium phosphate pH 7.0. Protein and lipid samples were incubated at 23°C for 1 hour before analysis. Five scans were collected for each sample using a Jasco J-720 circular dichroism spectropolarimeter, at a scan rate of 20 nm min⁻¹, 1 nm resolution, 20 mdeg sensitivity, and 1 mm path length.

Steady-state fluorescence spectroscopy

Lipid vesicles were created with DOPC and DOPG at a 60:40 ratio. Reference tryptophan emission spectra were collected for 1 μ M protein and 200 μ M lipid (1:200 protein:lipid ratio), in a 5 mm cuvette buffered by 50 mM potassium phosphate pH 7.0. Protein and lipid samples were incubated at 23°C for 2 hours before analysis. Two scans were collected for each sample (average of 5 points per wavelength) using an SLM-48000 spectrofluorometer (SLM-AMINCO, Urbana, IL). The excitation wavelength was 295 nm, monochromator slits 2 nm; the emission scan was from 305 to 500 nm, monochromator slits 16 nm. Polarizers were oriented to provide horizontal excitation and vertical emission to minimize light scattering from lipid vesicles.

To determine the mole fraction partition coefficient (K_x) of CED-9, 0.5 μ M protein was incubated with lipid ranging from 0.5 to 250 μ M; for CED-9 lacking the C-terminal transmembrane domain, 1 μ M protein was incubated with lipid ranging from 1 to 500 μ M. Tryptophan emission spectra were collected under similar conditions used for reference spectra with the exception that emission was monitored at the region of maximum fluorescence intensity (333–337 nm). The fraction of protein (f_p) associated with lipids at a given lipid concentration was estimated as follows:

$$f_p = \frac{(f_{\text{intensity}} - f_0)}{(f_{\text{max}} - f_0)}$$

where f_0 , $f_{\text{intensity}}$, and f_{max} are the initial tryptophan fluorescence before the addition of lipid, tryptophan fluorescence at a given lipid concentration, and the maximum tryptophan fluorescence, respectively. This data was used to determine K_x values using Igor Pro (Wavemetrics, Inc.) and the following equation:²⁰

$$f_p = \frac{K_x[L]}{[W] + K_x[L]}$$

where $[W]$, $[L]$ and f_p are the concentration of water, the concentration of lipid, and the fraction of protein associated with lipids at a given concentration of lipid, $[L]$.

We note that precise interpretations of fluorescence changes are not possible due to the presence of multiple tryptophan residues in both CED-9

constructs (six in full-length CED-9, five in CED-9 lacking the C-terminal transmembrane domain).

Vesicle leakage assay

8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and *p*-xylene-bis-pyridinium (DPX) were obtained from Molecular Probes (Eugene, OR). Lipid vesicles were created with DOPC and DOPG at a 60: 40 ratio. Dried lipids were resuspended at 10 mg/mL in 10 mM HEPES pH 7.0, 50 mM KCl, 12.5 mM ANTS and 45 mM DPX. After five freeze-thaw cycles, lipids were extruded and unencapsulated dyes were removed using a PD-10 desalting column (Amersham). The phospholipid concentration was measured using an ammonium ferrothiocyanate assay.⁴⁹

Vesicle leakage assays were performed by incubating 100 μ M lipid with varying concentrations of protein in 50 mM potassium phosphate pH 7.0 at 23°C for 2 hours. ANTS fluorescence of each sample was measured in a 5 mm cuvette; the excitation wavelength was 355 nm, monochromator slits 2 nm; the emission wavelength was 520 nm, monochromator slits 16 nm. The amount of vesicle leakage was determined by the following equation:

$$\text{leakage \%} = \frac{(f_{\text{intensity}} - f_0)}{(f_{\text{max}} - f_0)}$$

where f_0 , $f_{\text{intensity}}$ and f_{max} are the initial ANTS fluorescence before the addition of protein, ANTS fluorescence at a given protein concentration, and ANTS fluorescence after disruption of vesicles by Triton-X-100 (final concentration 0.2% (v/v)), respectively.

Vesicle lipid mixing assay

Lipid vesicles were created with DOPC, DOPG, rhodamine labeled DOPE (Rh-DOPE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl (NBD) labeled DOPE (NBD-DOPE) at a 58.8:39.2:1:1 molar ratio. These vesicles were mixed with unlabeled vesicles at a ratio of 1:9 immediately before use. Lipid mixing assays were performed by incubating 100 μ M of total lipid with varying concentrations of protein in 50 mM potassium phosphate pH 7.0 at 23°C for 3 hours. NBD fluorescence of each sample was measured in a 5 mm cuvette; the excitation wavelength was 450 nm, monochromator slits 2 nm; the emission wavelength was 530 nm, monochromator slits 16 nm. The amount of vesicle lipid mixing was determined by the following equation:

$$\text{mixing \%} = \frac{(f_{\text{intensity}} - f_0)}{(f_{\text{max}} \times q - f_0)}$$

where f_0 , $f_{\text{intensity}}$, f_{max} and q are the initial NBD fluorescence before the addition of protein, NBD fluorescence at a given protein concentration, NBD

fluorescence after vesicle disruption by Triton-X-100 (final concentration 0.2% (v/v)), and a correction factor for the decrease in NBD quantum yield by Triton-X-100, respectively. A correction factor of 1.75 was determined by measuring the fluorescence of unquenched concentrations of NBD in the presence and absence of Triton-X-100, as previously described.⁴⁶

Dynamic light scattering

Lipid vesicles were created with DOPC and DOPG at a 60: 40 ratio. The size distribution of vesicles was determined for 1 μ M protein and 200 μ M lipid (1:200 ratio) buffered with 20 mM sodium acetate and 20 mM potassium phosphate. Reactions were incubated for 2 hours at 23°C, and then autocorrelation spectra were acquired using a Malvern ZetaSizer Nano ZS90 equipped with a 633 nm laser. Data were analyzed using two different algorithms implemented in the Dispersion Technology Software (Malvern). The cumulant algorithm was used to model data assuming a monomodal species, while the “General Purpose” regularization algorithm was used to resolve different sized populations of vesicles.

Electron microscopy analysis

Lipid vesicle samples were adsorbed onto ionized, formvar-carbon coated grids and subsequently negatively-stained with 0.4% uranyl acetate supplemented with 1% trehalose for support. These stained samples were analyzed on a Philips EM 420 TEM, and images were collected using a Soft Imaging System Megaview III digital camera. Images are representative of at least two separate experiments for each condition examined.

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