Mitochondria Localization and Dimerization Are Required for CIDE-B to Induce Apoptosis*

Received for publication, March 28, 2000, and in revised form, June 2, 2000
Published, JBC Papers in Press, June 2, 2000, DOI 10.1074/jbc.C000207200
Zhengming Chen, Ke Guo, Shen Yon Toh, Zhihong Zhou, and Peng Li‡
From the Laboratory of Apoptosis Regulation, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Singapore

Cell death-inducing DFF45-like effector (CIDE)-B is a member of the novel family of apoptosis-inducing factors that share homology with the N-terminal region of DFF, the DNA fragmentation factor. The molecular mechanism of CIDE-B-induced apoptosis is unclear. We have shown here that CIDE-B protein is localized in mitochondria and forms homodimers and heterodimers with other family members. Serial deletion analyses suggest that the mitochondria localization signal and dimerization interface are overlapped and localized to the 30 amino acid residues at the C-terminal region of CIDE-B. Mitochondria localization and dimerization are both required for CIDE-B-induced apoptosis. Our study has thus revealed a mechanism for CIDE-B-induced apoptosis by localization to mitochondria and the formation of a high affinity homo- or heterodimeric complex.

Mitochondria are major organelles that respond to death stimuli by releasing factors such as cytochrome c and apoptosis-inducing factor and altering the cellular reduction-oxidation (redox) potential and oxidative phosphorylation (1–3). A number of pro-and anti-apoptotic proteins reside in mitochondria including various caspases (4), the ced-4 and ced-9 (5) Bcl-2 family proteins (6), and the Nix family proteins (7, 8). Mitochondria localization is important for the anti-apoptotic activity of Bcl-2 (6) or the pro-apoptotic activity of Nix (7, 8), as deletion of the C-terminal mitochondria localization signal abrogates their activity. Many of the Bcl-2 family members can form either homodimers or heterodimers with other family members (6, 9, 10). Activation of pro-apoptotic Bax appears to induce subcellular translocation from cytosol to mitochondria as well as homodimerization (11). Mutational analyses have revealed that the conserved BH3 domain of the pro-apoptotic Bcl-2 family members such as Bax plays an important role in mediating homo- or heterodimerization (6).

The DNA fragmentation factor (DFF)1 (12, 13) consists of two subunits, a nuclease (CAD/DFF40) and its inhibitor (DFF45/ICAD). The N-terminal domain of DFF45 is required for its chaperone function by associating with the N-terminal region of DFF40 (14, 15). A novel family of cell death-inducing DFF45-like effectors (CIDEs) was identified by its high homology with both of the N-terminal domains of DFF40 and DFF45 (16, 17). CIDE proteins can be divided into the N-terminal CIDE-N domain, which shares homology with DFF40/45, and the C-terminal CIDE-C domain, which shares homology within CIDE proteins only (16). Although this class of proteins shares homology with DFF45/40 at the N-terminal region, their functions differ significantly. Unlike DFF45, over-expression of CIDEs in mammalian cells shows strong cell death-inducing activity with the C-terminal domain (CIDE-C) being sufficient for its cell death activity (16). We have recently participated in solving the structure of the N-terminal domain of CIDE-B (or CIDE-N) (18). Our structural analyses suggested that CIDE-N domains interact with each other with low affinity and that the binding surface has a novel bipolar property consisting of two oppositely charged regions. This novel homophilic association strongly suggests that CIDE-N domain is a weak interaction interface functioning as a regulatory domain for CIDE-B protein (18).

To gain insight into the molecular mechanism by which CIDE-B induces apoptosis, we characterized its subcellular localization. We observed that CIDE-B proteins are mitochondrially localized and form homodimers and heterodimers with other family members. Systematic deletion analysis showed that the C-terminal region of CIDE-B is responsible for its mitochondria localization and dimerization. Our data also suggest that both mitochondria localization and high affinity interaction are required for CIDE-B-induced apoptosis. Therefore, CIDE proteins form a novel family of pro-apoptotic mitochondrial proteins that require dimerization to function.

MATERIALS AND METHODS

Construction of Expression Plasmids—The full-length and truncated forms of CIDE-B were PCR-amplified using specific primers corresponding to the regions described in Fig. 2A. All of the PCR products contained NcoI at the 5’-end and BamHI at the 3’-end. The PCR products were digested with NcoI and BamHI and then were inserted in-frame into pBluescript KS-Flag and pBluescript KS-HA to generate the 5’-Flag- or HA-tagged fragments. The Flag- and HA-tagged inserts were then digested with HindIII and XbaI and subcloned into pCMV5 vector to produce the mammalian expression constructs. To make the GFP-tagged proteins, fragments were blunted at the NcoI site and subcloned into pEGFP-C3 (CLONTECH) at the XhoI and BamHI sites. The validity of all the constructs was confirmed by DNA sequencing.

Transient Transfection, Immunoprecipitation, and Western Blotting—6×10⁵ human embryonic 293T cells were seeded in 60-mm dish for 24 h prior to transfection. Cells were co-transfected using Doser liposomal transfection reagent (Roche Molecular Biochemicals) following the manufacturer’s instruction. For Co-immunoprecipitation experiments, 4 μg of Flag- and GFP-tagged CIDE-B and 24 μg of Doser were added to one 60-mm dish. Cells were washed once with ice-cold PBS and harvested in 300 μl of Triton X-100 lysis buffer (10 m M Tris (pH 7.4), 150 m M NaCl, 10 m M EDTA, 10 m M EGTA, and 1% Triton X-100 containing 1 m M phenylmethylsulfonyl fluoride, 10 m M dithiothreitol, 5 μg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin) 20 h after transfection. The cell lysate was incubated for 10 min on ice, sonicated, and centrifuged for 30 min at 15,000 × g at 4 °C. A 50-μl aliquot of supernatant was mixed with 50 μl of 2× SDS loading buffer, and 10 μl of the mixture was subjected to 15% SDS-polyacrylamide gel electro-
phoresis to test the expression of protein. The remaining supernatant was incubated with 10 μl of anti-Flag M2-agarose affinity gel (Sigma) for 4 h at 4°C. After washing three times with Triton X-100 lysis buffer, the beads were boiled in 50 μl of 1× SDS loading buffer and subjected to 15% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to Hybond C membranes and immunoblotted with rabbit polyclonal anti-OctA, anti-HA, and anti-GFP antibodies (all from Santa Cruz Biotechnology), respectively. The proteins were detected by horse-radish peroxidase-conjugated secondary antibody (Bio-Rad) and visualized by Super Signal reagent (Pierce).

Cell Death Assay—3 × 10⁵ CHO cells were transfected with 0.5 μg of the reporter plasmid (pCMV β-galactosidase) plus 3 μg of CIDE-B expression plasmid in a 6-well tissue culture dish using Dsper. 24 h post-transfection, cells were fixed in 4% paraformaldehyde-PBS for 20 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (SIGMA) at 37 °C for 30 min. Approximately 300 blue cells were counted for each transfection (n = 4) from random fields under phase contrast microscope. Apoptotic cells were distinguished based on morphological alteration typical of adherent cells undergoing apoptosis including becoming rounded, condensed, and detached from the dish. The mean of this calculation was used to calculate the percentage of apoptosis.

Mitochondria Localization—3 × 10⁵ COS-1 cells were transfected with 3 μg of GFP-CIDE-B or its truncations on a 22 × 22-mm coverslip using Dsper. 23 h post-transfection, cells were incubated with 100 nM MitoTracker (Molecular Probes) for 30 min at 37 °C. Treated cells were fixed with 4% paraformaldehyde-PBS for 20 min at room temperature and washed three times with PBS. Cells were permeabilized in 0.2% Triton X-100 for 10 min, washed three times in 0.1% Triton X-100-PBS, rinsed three more times with PBS, and visualized using a confocal microscope.

RESULTS AND DISCUSSION

To characterize the subcellular localization of CIDE-B, the GFP coding region was fused to the N-terminal ATG of human CIDE-B and its deletion mutants. Black boxes represent the conserved regions between CIDE-B and other family members. A, schematic diagram of hCIDE-B and its deletion mutants. B, homodimerization of CIDE-B. IP, immunoprecipitation using monoclonal antibodies against Flag; IB, immunoblot using rabbit anti-HA antibodies or rabbit anti-OctA antibodies (Santa Cruz). The negative control is Flag-tagged Jun N-terminal kinase proteins. C, homodimeric interaction of CIDE-B is mediated by the C-terminal region of CIDE-B (CIDE-BΔN), D, the heterodimeric interaction between CIDE-B and CIDE-A is also dependent on the C-terminal region of CIDE-B.
anti-apoptotic proteins, thereby inducing apoptosis. It is also
with Bcl-2 family proteins such as Bcl-2 and Bcl-XL to chelate
By localizing to mitochondria, CIDE-B may interact directly
exerts its cell death function by localization to mitochondria.
may help CIDE-B to insert into the mitochondria membrane.
located at the C terminus of CIDE-B (residues 166–195). The GFP fusion proteins of CIDE-ΔN and its truncations were co-transfected with Flag-CIDE-B. The co-immunoprecipitated products (IP) were detected with GFP antibodies. IB, immunoblot. B, the relative binding affinity between CIDE-ΔN and its truncations. The intensity of Flag-CIDE-B protein and co-immunoprecipitated products for each mutant was measured by densitometer scan (Bio-Rad) and normalized to deduce the fold difference between CIDE-ΔN and the minimal region required for interaction (CIDE-B166–195).

FIG. 3. Serial deletion analyses to show the CIDE-B homodimeric interaction interface. A, the homodimeric interaction interface is located at the C terminus of CIDE-B (residues 166–195). The GFP fusion proteins of CIDE-ΔN and its truncations were co-transfected with Flag-CIDE-B. The co-immunoprecipitated products (IP) were detected with GFP antibodies. IB, immunoblot. B, the relative binding affinity between CIDE-ΔN and its truncations. The intensity of Flag-CIDE-B protein and co-immunoprecipitated products for each mutant was measured by densitometer scan (Bio-Rad) and normalized to deduce the fold difference between CIDE-ΔN and the minimal region required for interaction (CIDE-B166–195).

toTracker (data not show). Therefore, our data strongly indi-
cate that CIDE-B is localized to the mitochondria.
The region(s) that is responsible for CIDE-B mitochondria
localization was further defined by generating GFP fusion pro-
teins containing truncated forms of CIDE-B (Fig. 2A). GFP
fusion proteins containing the N-terminal region of CIDE-B
(CIDE-BAC) showed a diffused pattern in both nucleus and
cytoplasm. In contrast, GFP fused to the C-terminal region of
CIDE-B (CIDE-BAN) was found as spherically shaped granules
and was localized predominantly to mitochondria (Fig. 1).
Therefore, the mitochondria localization signal appears to re-
side in the C-terminal region of CIDE-B. GFP fusion proteins
with a deletion of 30 residues from the N-terminal end of this
C-terminal domain (CIDE-B-(148–219)) were still localized to
mitochondria. However, deletion of the C-terminal 39 residues
from the C-terminal domain (CIDE-B-(118–180)) disrupted the
mitochondria localization of GFP fusion proteins, suggesting
that C-terminal is required for mitochondria localization. Sur-
prisingly, GFP fusion proteins containing the C-terminal 39
residues of CIDE-B alone (CIDE-B-(181–219)) were expressed
evenly throughout the cytoplasm and nucleus and were not
localized to any organelle-like structure. GFP fusion proteins
containing CIDE-B regions from 118 to 148 (CIDE-B-(118–
148)) or 148 to 180 (CIDE-B-(148–180)) did not show typical
mitochondria localization either. To explore the possibility
that disruption around amino acid 180 may result in abolishing
the mitochondria localization signal, we generated constructs that
span the region near amino acid 180. Indeed, GFP fusion pro-
teins containing regions from residue 166–219 (CIDE-B-(166–
219)) or 148–195 (CIDE-B-(148–195)) all displayed mitochondria
localization. The minimal region tested that showed
mitochondria localization by GFP analysis consists of amino
acid 166–195 (CIDE-B166–195, Fig. 1), indicating that residues
166–195 at the C-terminal domain of CIDE-B are necessary
and sufficient to target CIDE-B to mitochondria. Computer
analysis of the minimal mitochondria localization signal re-
vealed that it contains a long stretch of α-helical structure that
may help CIDE-B to insert into the mitochondria membrane.

Cell death stimuli such as staurosporine and etopside did
not induce GFP-CIDE-B translocation from mitochondria
to the nuclei or cytosol (data not shown), suggesting that CIDE-B
exerts its cell death function by localization to mitochondria.
By localizing to mitochondria, CIDE-B may interact directly
with Bcl-2 family proteins such as Bcl-2 and Bcl-XL to chelate
anti-apoptotic proteins, thereby inducing apoptosis. It is also
possible that CIDE-B may directly induce mitochondria change
such as disruption of mitochondria membrane potential, induc-
tion of the production of reactive oxygen species, and the re-
lease of apoptosis-inducing factors.

Our previous biochemical and structural analyses suggested
that the N-terminal region of CIDEs acts as a weak interaction
interface through homophilic interaction, whereas synergistic
and high affinity binding requires the contribution of other
domains (14, 18). To explore the possibility that CIDE proteins
also contain multiple interaction domains, Flag-tagged CIDE-B
was co-expressed with HA-tagged CIDE-B or CIDE-A in 293T
cells. Flag-CIDE-B was immunoprecipitated with monoclonal
antibodies against Flag epitope, and the co-precipitating pro-
tein was detected with HA antibodies. HA-CIDE-B was co-
precipitated with Flag-CIDE-B but not with control proteins
(Fig. 2B), indicating a strong interaction of CIDE-B with itself.
Although we cannot exclude formation of high order oligomers,
we assume and will refer to this interaction as dimerization.
The HA-tagged C-terminal region of CIDE-B (HA-CIDE-BAN)
but not the N-terminal region (HA-CIDE-BΔC) was co-precipitated
with Flag-CIDE-B, indicating that the C-terminal region of
CIDE-B mediates stable dimer formation. This result was
further confirmed by cotransfecting HA-CIDE-BAN with Flag-
CIDE-BAN and Flag-CIDE-BΔC, respectively. HA-CIDE-BAN
was co-immunoprecipitated only with Flag-CIDE-BAN and not

FIG. 4. Apoptosis induced by hCIDE-B and its deletion mu-
tants. 3 μg of indicated plasmids and 0.5 μg of pCMV-β-galactosidase
were transfected into CHO cells. Apoptotic cells were quantitated, and
the percentage of cell death was calculated against the total number of
β-galactosidase-positive cells. The error bars show a standard deviation
determined from at least four independent measurements. The inset is
a Western blot against GFP antibody showing the expression levels of
GFP-CIDE-B and its truncations used in the cell death assay.
with Flag-CIDE-BΔC (Fig. 2C). Reciprocal experiments using HA antibodies to immunoprecipitate the complex and detecting the co-precipitated product with Flag antibodies yielded the same results (data not shown). Similar experiments conducted between HA-CIDE-B and Flag-CIDE-A suggested that CIDE-B interacts with CIDE-A (Fig. 2D), mediated by the C-terminal region. Consistent with our previous data, CIDE-BΔC/ CIDE-BΔAC interaction (Fig. 2C) was weak and not detectable by co-immunoprecipitation experiments (14, 18).

To further delineate regions within the C-terminal domain of CIDE-B that are required for dimerization, we cotransfected the GFP-tagged CIDE-C domain and various truncated forms with Flag-tagged CIDE-B into 293T cells. The Flag antibody-immunoprecipitated products were probed using GFP antibodies to detect the co-precipitated proteins. In agreement with the results shown above, the CIDE-BΔN domain was found bound to CIDE-B proteins (Fig. 3A, lane 2). Deletion of the N-terminal 30 amino acids of the CIDE-BΔN domain had little effect on the homodimeric interaction, suggesting that amino acid residues 148–219 contain the dimerization interface (Fig. 3A, lane 3). Deletion of the C-terminal 39 amino acids almost completely abolished the homodimeric interaction (Fig. 3A, lane 4), suggesting that this region is important in mediating the CIDE-B/CIDE-B interaction. GFP fusion proteins containing the C-terminal 39 amino acids of CIDE-BΔN alone, the middle region (residue 148–180), or the N-terminal region (residue 118–148) alone showed no binding activity to CIDE-B (Fig. 3A, lanes 5–7). To explore the possibility that disruption at residue 180 may disrupt the interaction interface, we tested the ability of GFP fusion proteins containing residues 166–219, 148–195, and 166–195 of CIDE-C domain to co-immunoprecipitate with Flag-CIDE-B. Indeed, GFP fusion proteins containing these regions all showed weak binding activity to CIDE-B (Fig. 3A, lanes 8–10). These results (Fig. 3A) suggest that the minimal region required for CIDE-B/CIDE-B interaction is from residues 166–195 in the C-terminal region of CIDE-B. Quantitative analysis suggested that CIDE-BΔN and CIDE-B-(148–219) showed much higher binding affinity to CIDE-B (27- or 20-fold higher amounts co-immunoprecipitated, respectively) compared with CIDE-B-(166–195) (minimal region required for the interaction, Fig. 3B). These data also suggested that residues upstream or downstream of residues 166–195 are required for high affinity CIDE-B/CIDE-B interaction. Interestingly, the dimerization interface coincides with the mitochondria localization signal. Dimerization (or higher order oligomerization) of CIDE-B may increase the local concentration of this protein and help it to target to mitochondria and effectively induce cell death.

As mitochondria are the major organelles mediating cell death signals and activating the cell death machinery and dimerization is critical for the function of many cell death proteins, we tested whether mitochondria localization and dimerization of CIDE-B are required for CIDE-B-induced apoptosis. The N-terminal domain of CIDE-B (CIDE-BΔC) showed no cell death effect, whereas the C-terminal domain (CIDE-BΔN) alone showed a level of cell death comparable to full-length CIDE-B (70 and 65%, respectively). A deletion of the N-terminal end of the CIDE-BAN (CIDE-B-(148–219)) that showed mitochondria localization and high affinity interaction was still effective in inducing apoptosis (55%). CIDE-B with a deletion of the C-terminal 39 residues (CIDE-B-(118–180)), which neither localized to mitochondria nor dimerized with itself, showed no apoptotic activity (20%). Truncations of CIDE-B protein (CIDE-B-(118–148), -(148–180), and -(181–219)), which showed no mitochondria localization, all showed no activity in triggering apoptosis. CIDE-B deletions that showed mitochondria localization but had low affinity interaction (CIDE-B-(166–219), -(148–195), and -(166–195)) all demonstrated weak apoptotic activity slightly higher than the vector but significantly lower than that of CIDE-BAN domain and CIDE-B-(148–219). The differences in cell death effect is not due to protein expression levels, as CIDE-B and its truncation mutants all showed a similar level of expression (Fig. 4, inset). Our data thus suggest that the C-terminal domain of CIDE-B plays an important role for CIDE-B-induced apoptosis by directly targeting CIDE-B to mitochondria, mediating CIDE-B/ CIDE-B interaction, and inducing apoptosis. It is very interesting to identify the 30 amino acids (from 166 to 195) at the C-terminal region of CIDE-B as both the mitochondria localization signal and the interaction interface. Single-point mutations at this region that abolish one of the activities would be useful in distinguishing these roles.

Acknowledgments—We are grateful to Lai Ping Yaw for providing valuable reagents and technical help. We also thank Dr. John McCarty for critical comments on the manuscript.

REFERENCES
1. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
2. Li, P., Jihjawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
3. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
4. Porter, A. G. (1999) Trends Cell Biol. 9, 394–401
5. Chen, F., Hersh, B. M., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y., and Horvitz, H. R. (2000) Science 287, 1445–1449
6. Gross, A. G., McDonnell, J. M., and Korsmeyer, S. J. (1999) Genes Dev. 13, 1899–1911
7. Yasuda, M., Theodorakis, P., Subramanian, T., and Chinnadurai, G. (1998) J. Biol. Chem. 273, 12415–12421
8. Chen, G., Cizeau, J., Velde, C. V., Park, J. H., Bozek, G., Bolton, J., Shi, L., Dubik, D., and Greenberg, A. (1999) J. Biol. Chem. 274, 7–10
9. Ray, R., Chen, G., Vande, V. C., Cizeau, J., Park, J. H., Reed, J. C., Gietz, R. D., and Greenberg, A. H. (2000) J. Biol. Chem. 275, 1439–1444
10. Gross, A., Jockel, J., Wei, MC., and Korsmeyer, S. J. (1998) EMBO J. 17, 3878–3885
11. Hou, Y.T., Wolter, K. G., and Youle, R. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3668–3672
12. Enari, M., Sakahira, H., Okawa, O., Iwamatsu, A., and Nagata, S. (1998) Nature 391, 43–50
13. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) Cell (1997) 89, 175–184
14. McCarty, J. S., Toh, S. Y., and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 176–180
15. McCarty, J. S., Toh, S. Y., and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 181–185
16. Inohara, N., Koseki, T., Chen, S., Wu, X., and Nunez, G. (1998) EMBO J. 17, 2526–2533
17. Inohara, N., and Nunez, G. (1999) Cell Death Differ. 6, 823–824
18. Lugovskoy, A. A., Zhou, P., Chou, J. J., McCarty, J. S., Li, P., and Wagner, G. (1999) Cell 99, 747–755