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Identification and Characterization of a Dimerization Domain in CED-6, an Adapter Protein Involved in Engulfment of Apoptotic Cells*

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Phagocytosis of apoptotic cells is a key step in the completion of programmed cell death that occurs throughout life in multicellular organisms. The molecular events involved in clearance of apoptotic cells are just beginning to be elucidated. Recently, CED-6, an adapter protein involved in engulfment has been cloned in *Caenorhabditis elegans* and in humans. CED-6 is composed of a phosphotyrosine-binding (PTB) domain and a proline-rich C-terminal domain with no apparent catalytic domain. Since PTB domains, originally identified in Shc, mediate intracellular signaling downstream of cell surface receptors, CED-6 has also been proposed to mediate intracellular signals leading to engulfment. In this report, we demonstrate that CED-6 dimerizes through a leucine zipper domain that is immediately adjacent to the PTB domain. Several lines of evidence based on co-immunoprecipitation studies, yeast two-hybrid assays, and gel filtration studies suggest that CED-6 exists as a dimer *in vivo*. Through mutational analyses, we show that the leucine zipper is necessary and sufficient for CED-6 dimerization and that this dimerization is conserved among *C. elegans*, rodent, and human CED-6 proteins. We propose that dimerization may have unique implications for ligand binding via CED-6 and its function during the phagocytosis of apoptotic cells.

Apoptosis of specific cell populations and the subsequent phagocytosis of apoptotic cells are critical events that occur throughout the lifespan of organisms (1). Clearance of dead cells is mediated by professional phagocytes such as macrophages and dendritic cells, as well as by nonprofessional phagocytes within certain tissues (2–4). Removal of apoptotic cells occurs very rapidly *in vivo* and helps to prevent secondary necrosis and the release of potentially harmful contents from dying cells (5). In humans, the failure to properly remove apoptotic cells has been implicated in certain types of chronic inflammation. Engulfment of apoptotic cells is mediated by the recognition of specific markers on dying cells by receptors on the phagocytic cells (6, 7). The subsequent intracellular signals delivered within the phagocyte through these recognition re-

ceptors are thought to mediate the cytoskeletal rearrangement and the uptake of apoptotic cells. Although several surface receptors involved in phagocytosis of apoptotic cells have been recognized, very little is known about intracellular signaling mediated by these receptors and the precise molecular events regulating the execution of engulfment (2, 4, 5).

Recently, genetic studies in *Caenorhabditis elegans* have identified at least six genes that are involved in clearance of apoptotic cells (1, 8). These six genes fall into two partially redundant complementation groups and include *ced-1*, *ced-6*, and *ced-7* in one group and *ced-2*, *ced-5*, and *ced-10* in another (9). The cloning of the *ced-6* gene by Liu and Hengartner (10) showed that the ceCED-6¹ protein resembles an adapter protein with an N-terminal phosphotyrosine-binding (PTB) domain, and a C-terminal proline-rich region with no apparent catalytic domain. The CED-6 PTB domain is highly homologous to the “classical” PTB domains originally identified in other mammalian signaling proteins such as Shc. PTB domains of proteins such as Shc, Numb, FE65, and X11 have been shown to mediate protein-protein as well as protein-phospholipid interactions (11–14). Because many PTB domain-containing proteins have been shown to function downstream of cell surface receptors, it was proposed that CED-6 may function as a signaling adapter protein during phagocytosis of apoptotic cells (10).

Recently, the human and *Drosophila* homologues of *ced-6* have also been cloned (15, 16). Alignment of the amino acid sequences of *C. elegans*, *Drosophila*, and human CED-6 proteins demonstrates that the N-terminal PTB domain is evolutionarily conserved, whereas the C-terminal proline-rich sequences are less well conserved (16). Since the overall domain organization of these proteins are the same, this suggested an evolutionarily conserved function for CED-6. Consistent with this, overexpression of hCED-6 in J774 cells led to enhanced phagocytosis of apoptotic cells (16). In addition, expression of hCED-6 partially rescued the defect in apoptotic cell clearance in worms lacking a functional ceCED-6 protein (15). Although these data clearly suggest a role for hCED-6 in engulfment of apoptotic cells, precisely how CED-6 functions during engulfment is not yet understood. At present, the ligands of CED-6 are not known. It is believed that CED-6, via its PTB domain, may interact with ligands (most likely at the membrane) and that through other protein-protein interactions, via its C ter-

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¹ The abbreviations used are: ceCED-6, *C. elegans* CED-6; hCED-6, human CED-6; PTB, phosphotyrosine-binding; LZ, leucine zipper domain; GST, glutathione S-transferase; HA, hemagglutinin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GSH, glutathione-Sepharose; AECSEF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

minus, CED-6 would mediate downstream signaling leading to phagocytosis of apoptotic cells.

In this report, we identify and characterize a leucine zipper domain immediately C-terminal to the CED-6 PTB domain. Several lines of evidence indicate that this leucine zipper (LZ) domain mediates dimerization of CED-6 and that the LZ-dependent dimerization is evolutionarily conserved in CED-6 proteins from worm to humans. Since such leucine zipper domains are not seen in other proteins containing PTB domains such as Shc, this dimerization is likely to have unique implications for ligand binding via CED-6 and its signaling function during engulfment of apoptotic cells.

EXPERIMENTAL PROCEDURES

Plasmids—All hCED-6 constructs were obtained by PCR and cloned into the eukaryotic expression vectors pEBG (for expression as a glutathione *S*-transferase (GST) fusion protein) and pEBB-HA (for expression of hemagglutinin (HA) tagged proteins) using 5'-*Nde*I and 3'-*Not*I restriction sites (17). The original template for *hced-6* has been described (16). The *Nde*I site within the coding sequence of the original *hced-6* clone was destroyed by a silent mutation using a PCR-based mutagenesis strategy. The internal primers used were 5'-GAT AAA ACT GAC AAG AGG ATA TTC ACT TTC ATT TGC AAA GAT TCT GAG TCA AAT AAA CAT TTG-3' and 5'-CAA ATG TTT ATT TGA CTC AGA ATC TTT GCA AAT GAA AGT GAA TAT CCT CTT GTC AGT TTT ATC-3'. This construct was used as the template for all further hCED-6 constructs. Mutation of the central prolines (L174P/L183P) were generated using a PCR-based approach with internal primers 5'-GAA TCC AAG ACC CCG AAA CAG AAA ATA TGG AAC CCA AAA AT-3' and 5'-CTT GTA CTT TAT TTT TGG GTT CCA TAT TTT CTG TTT CGG GGT CTT G-3'. The *C. elegans ced-6* in the pEBG and pEBB-HA plasmids were generated by PCR using the cDNA that has been previously described (10). *ceCED-6* was also cloned into pEBG and pEBB-HA vectors using the 5'-*Nde*I and 3'-*Not*I sites. The leucine to proline mutations (L215P and L208P/L215P) were carried out using a similar approach as described above for human CED-6 using 5'-AAG AAA AAG ATT GTG GAG CCT GAA ACC GAG AAT C-3' to mutate leucine 215 and 5'-GCT CCA CAA TCT TTT TCT TTG GAA TGT ATA TTT GCT TC-3' to mutate leucine 208. All regions that were cloned by PCR were sequenced to ensure fidelity.

Cell Culture and Transfection—Human embryonic kidney 293T cells and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum, 5% fetal calf serum, and penicillin/streptomycin/glutamine (Life Technologies, Inc.). The Chinese hamster ovary fibroblast cell line LR73 (18) was cultured in Alpha-modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin/L-glutamine. 293T cells were transfected by the calcium phosphate method (5 Prime → 3 Prime, Inc., Boulder, CO). COS-7 cells were transfected using Superfect (Qiagen, Valencia, CA) according to manufacturer's recommendation. All transient transfections were performed using 2–5 μ g of the appropriate expression plasmid. LR73 cells were stably transfected by the calcium phosphate method. Briefly, 20 μ g of the appropriate expression plasmid (linearized with *Sph*I), was co-transfected with 0.5 μ g of puromycin resistance gene-expressing pAuro plasmid (linearized with *Not*I), as a selection marker. Cells were plated at different concentrations in growth media containing 7 μ g/ml of puromycin, and single clones were isolated for further studies.

Immunoprecipitations and Immunoblotting—Cells were lysed on the tissue culture plates with lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 10 μ M each aprotinin, leupeptin, pepstatin, AEBSF, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% Nonidet P-40). GST-tagged proteins were precipitated with glutathione-Sepharose (Amersham Pharmacia Biotech). Anti-HA immunoprecipitations were performed with using 12CA5 supernatant or H-7 anti-HA antibodies pre-bound to protein A or protein A-protein G-agarose conjugates (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Precipitates were washed four times with buffer containing 20 mM Tris, pH 7.6, 150 mM NaCl, 1 μ M each aprotinin, leupeptin, pepstatin, AEBSF, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.1% Nonidet P-40. Antibodies for Western blotting HA and GST-tagged proteins were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech. Antibody EP990445 to hCED-6 was generated by immunizing rabbits with a peptide corresponding to amino acids 31–44 from hCED-6. Monoclonal anti-Grb2 and anti-Shc

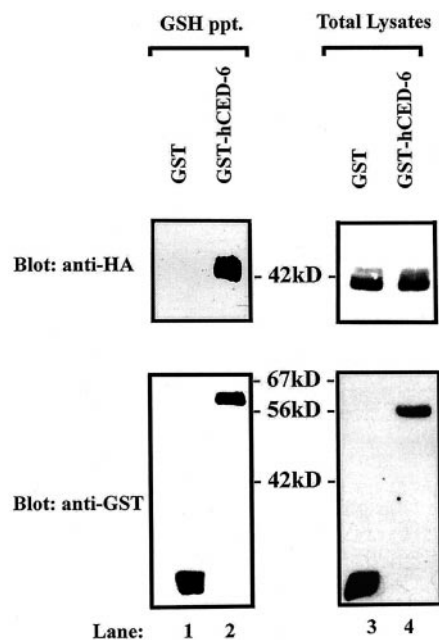


FIG. 1. Co-precipitation hCED-6 carrying two different tags. Plasmids encoding GST-tagged hCED-6 or the GST tag alone were co-transfected with HA-tagged hCED-6 into COS-7 cells. Following GSH precipitation, the bound proteins were separated by SDS-PAGE and immunoblotted with anti-HA antibodies (left upper panel). Similar amounts of GST alone and GST-hCED-6 were precipitated in both lanes (left lower panel) as determined by anti-GST immunoblotting. Comparable expression of HA and GST-tagged proteins in the transfected COS cells was confirmed in total cell lysates (right panels, lanes 3 and 4).

antibodies were obtained from Transduction Labs (Lexington, KY). All immunoblots were developed using enhanced chemiluminescence (Pierce).

Two-hybrid Assay—The two-hybrid system was purchased from CLONTECH Laboratories (Palo Alto, CA). *hced-6* was cloned in frame of the GAL4 DNA-binding domain (pAS2) and GAL4 activation domain (pGAD424) as a *Nde*I-*Sal*I fragment. The cloning was verified by DNA sequencing. Transformation of the *Saccharomyces cerevisiae* strain HF7c was done by the lithium acetate method according to the manufacturer's directions. Yeast colonies carrying putative interacting proteins were selected by growth on synthetic minimal media lacking tryptophan, leucine, adenine, and histidine in the presence of 5 mM 3-amino-1,2,4-triazole (Sigma) and by screening for β -galactosidase activity in a filter assay using as a substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Saxon Biochemicals, Hanover, Germany).

Gel Filtration—Proteins were separated on a 20-ml column of Sephadex G-75 (Sigma) equilibrated with 20 mM Tris-HCl, pH 7.6. 2×10^7 LR73 cells were lysed in 200 μ l of lysis buffer, filtered through a 0.22- μ m spin filter (Fisher), and loaded onto the column. The first 4 ml of void volume was discarded. 50 fractions containing 500 μ l were collected. Fractions were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

RESULTS

Dimerization of hCED-6—As part of our studies to understand human CED-6 function during phagocytosis of apoptotic cells, we generated hCED-6 constructs carrying two different epitope tags (HA-tagged or GST-tagged hCED-6) and expressed them transiently in COS-7 cells. Following glutathione-Sepharose (GSH) precipitation, we observed that the HA-tagged hCED-6 was precipitated with GST-tagged hCED-6 as determined by anti-HA immunoblotting (Fig. 1). In the same experiment, the GST tag alone did not precipitate the HA-hCED-6. Conversely, immunoprecipitation of HA-tagged hCED-6 (with anti-HA antibodies) co-precipitated the GST-tagged hCED-6 (see Fig. 5 below). These data suggested that hCED-6 proteins can form dimers/oligomers in cells. Expression of these proteins in 293T cells yielded similar results (data not shown).

TABLE I
Interaction of hCED-6 in a yeast two-hybrid assay

The cloning of hCED-6 into the yeast two-hybrid plasmids and the screening procedure for hybrid formation are described under "Experimental Procedures." The histidine auxotrophy was assessed by growth on histidine deficient media, and the β -galactosidase reporter gene activity was measured by a filter assay using X-gal. The p53 and SV40-Large T antigen fusions were provided as controls by the manufacturer. The dimerization between hCED-6 and the resultant interaction between the GAL4 DNA-binding and activation domains provided the His auxotrophy and β -galactosidase activity. The hCED-6 binding to itself was not due to "stickiness" of hCED-6 fused to GAL4-AD or GAL4-BD, because this was not seen in several other two-hybrid screens against multiple libraries (data not shown).

GAL4-DNA-binding domain fusion	GAL4-activation domain fusion	Histidine auxotrophy	β -Galactosidase activity
hCED-6	None	–	–
None	hCED-6	–	–
hCED-6	hCED-6	+++	+++
p53	None	–	–
None	SV40-Large T Ag	–	–
p53	SV40-Large T Ag	++	++

We also tested hCED-6 for self-interaction in a yeast two-hybrid assay. hCED-6 was cloned into appropriate yeast two-hybrid plasmids and expressed as a fusion with the GAL4 DNA-binding domain (GAL4-BD) or GAL4 activation domain (GAL4-AD). After transformation of appropriate yeast strain with these plasmids, the ability of yeast to grow on histidine-deficient media and β -galactosidase enzyme activity were tested (both of which are dependent on the interaction between the two hCED-6 fusion proteins). As shown in Table I, although GAL4-BD or Gal4-AD alone fused to hCED-6 were not sufficient, co-expression of the two constructs provided His auxotrophy and β -galactosidase activity. These data again suggest that hCED-6 can also dimerize in the heterologous yeast system.

Identification by Sequence Analysis of a Conserved Leucine Zipper within CED-6—By aligning the predicted PTB regions of hCED-6 with Shc, we observed that the PTB domain of hCED-6 appears to end at amino acid 150 (which corresponds to amino acid 204 of human p52Shc, consistent with the C terminus of the Shc-PTB domain determined by NMR). However, comparison of CED-6 protein sequence among the different species indicated that a high degree of homology exists up to amino acid 200 of hCED-6. Interestingly, the region between amino acid 150 and 200 of hCED-6 contains four leucines that are spaced seven residues apart, a feature characteristic of leucine zippered coiled-coil domains (19). Previously, leucine zipper domains have been identified in many transcription factors and a few cytoplasmic proteins and have been found to play a role in dimerization/multimerization of proteins (19). Thus, we hypothesized that hCED-6 could dimerize/oligomerize through this leucine zipper like sequence.

To better characterize the putative LZ region, we analyzed the sequences of hCED-6 and ceCED-6 through the program, Multicoil (20). This program accurately predicts the oligomerization state of protein sequences based on the position of specific hydrophobic and charged amino acids as well as pairwise residue interactions. As shown in Fig. 2A, the Multicoil program predicted the existence of a coiled-coil domain capable of forming a parallel side-by-side homodimer in both hCED-6 and ceCED-6. This coiled-coil is immediately C-terminal to the PTB domain homology. Analysis of a number of other PTB domain-containing proteins such as Shc, Numb, and Dab-1 in the Multicoil program failed to show a similar coiled-coil motif in these other proteins. The alignment of the human and ceCED-6 leucine zipper region and the key features of the sequence are shown in Fig. 2B. Helical wheel representation of

the hCED-6 coiled-coil showed that the four heptad leucines align correctly in a side-by-side dimer of hCED-6 molecules and that specifically spaced hydrophobic residues create a hydrophobic surface that can mediate dimerization (Fig. 2C).

Leucine Zipper Region Is Necessary and Sufficient to Mediate Dimerization of hCED-6—To determine whether the predicted leucine zipper region is necessary and sufficient for dimerization of hCED-6, we generated a series of hCED-6 constructs with deletions or point mutations. These constructs are schematically shown in Fig. 3A. Based on the Multicoil prediction, the dimerization region extended between amino acids 147 and 202. We tested whether this region alone, expressed as a GST-tagged protein would be capable of dimerizing with HA-tagged full-length hCED-6. GST-tagged 147–202 was co-expressed with HA-hCED-6 in COS cells and precipitated with GSH beads, and the co-precipitation of HA-hCED-6 was analyzed by anti-HA immunoblotting. As shown in Fig. 3B, the 147–202 region alone was sufficient to dimerize with full-length hCED-6. Although the predicted dimerization sequence shows a shoulder of probability starting from amino acid 147, the region of highest likelihood is restricted to residues 162–200, which includes the heptad leucines. When we tested a GST-tagged protein encoding the region 162–202, this construct precipitated HA-hCED-6 as efficiently as GST-147–202 or GST-tagged full-length hCED-6. These data suggested that the predicted coiled-coil/leucine zipper region is sufficient to mediate dimerization between human CED-6 proteins.

Although the above data indicated that the 162–202 region is sufficient for dimerization, it did not rule out the possibility that other regions may also contribute to dimerization between full-length hCED-6 proteins. To address this, GST-tagged constructs containing only the leucine zipper (GST-LZ), the PTB alone (GST-PTB), or the C terminus alone (GST-C-term) were individually co-transfected with HA-hCED-6. Following GSH precipitation, GST-PTB or the GST-C-term failed to co-precipitate the HA-hCED-6 protein, whereas the GST-LZ was able to do so (Fig. 3C). As would be expected from these results, GST-PTB+LZ was also capable of dimerizing with HA-hCED-6 (Fig. 3B, lane 4).

To further characterize the importance of the leucine zipper region in the dimerization of the full-length hCED-6 and to determine the requirement for the leucines, we mutated the two central leucines to prolines (L176P/L183P) to disrupt the helical structure. Full-length GST-tagged hCED-6 containing the two point mutations (L176P/L183P) was co-expressed with HA-tagged hCED-6. The mutant failed to co-precipitate the HA-hCED-6, indicating that the dimerization with the wild type protein was abrogated by the L176P/L183P mutation (Fig. 3D). Taken together, the above data indicate that the leucine zipper region between amino acids 162–202 of hCED-6 is necessary and sufficient for dimerization.

The ceCED-6 Also Dimerizes through a Leucine Zipper Domain—Since a homologous leucine zipper region is also observed in the ceCED-6 sequence, we hypothesized that the dimerization of CED-6 via the leucine zipper may be evolutionarily conserved. To test this, we generated constructs encoding GST-tagged and HA-tagged ceCED-6 (Fig. 4A) and co-expressed them in 293T cells. Following immunoprecipitation with anti-HA antibodies, the co-precipitation of GST-ceCED-6 was analyzed by anti-GST immunoblotting. As shown in Fig. 4B, HA-tagged ceCED-6 precipitated the GST-tagged ceCED-6 but not the control GST-Shc. When one or more leucines within the leucine zipper domain were mutated to prolines (L215P (lane 4) and L208P/L215P (lane 5)), the co-precipitation of GST-tagged ceCED-6 with HA-tagged ceCED-6 was abolished.

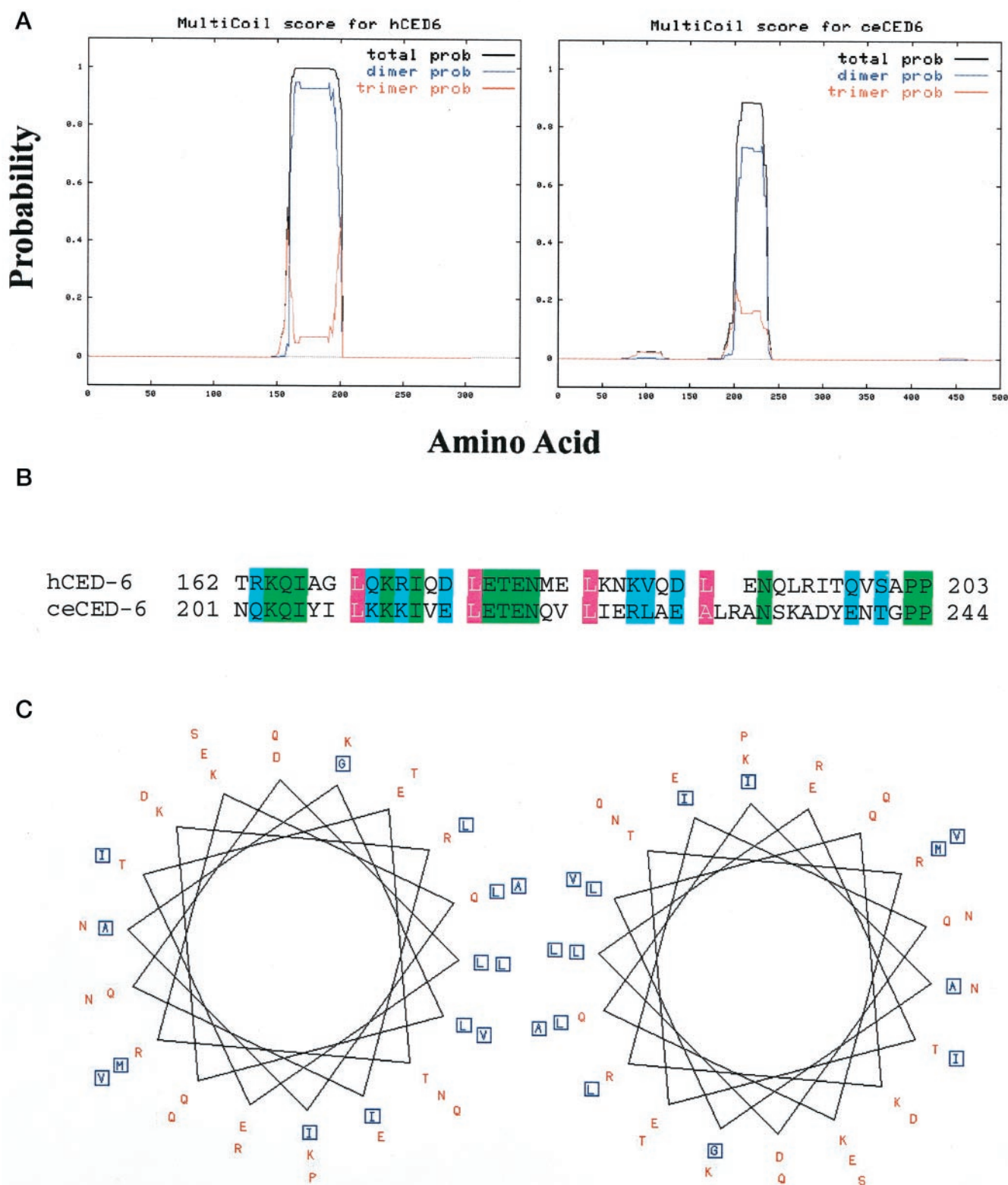


FIG. 2. The coiled-coil region of CED-6 is predicted to mediate dimer formation. A, the hCED-6 and ceCED-6 amino acid sequences were analyzed using the MultiCoil program, which predicts the presence of coiled-coils and the probability for dimer and trimer formation based on pairwise residue interactions. The probabilities for dimer (blue) and trimer (red) as well as overall score (black) are shown. Although the dimer probability is very high and considered significant, the trimer peaks at the shoulders of the dimer are due to artifacts of the algorithm and are not significant. B, alignment of the predicted dimerization regions of hCED-6 and ceCED-6 show heptad repeats with characteristically placed hydrophobic and charged residues. Identical residues are shown in yellow, and conserved residues are colored blue. The leucines forming the heptad repeat are shown in red. C, helical wheel prediction of hCED-6 leucine zipper shows that the critical leucines and many hydrophobic residues align on one side of the wheel to form a dimer interface with another hCED-6 molecule.

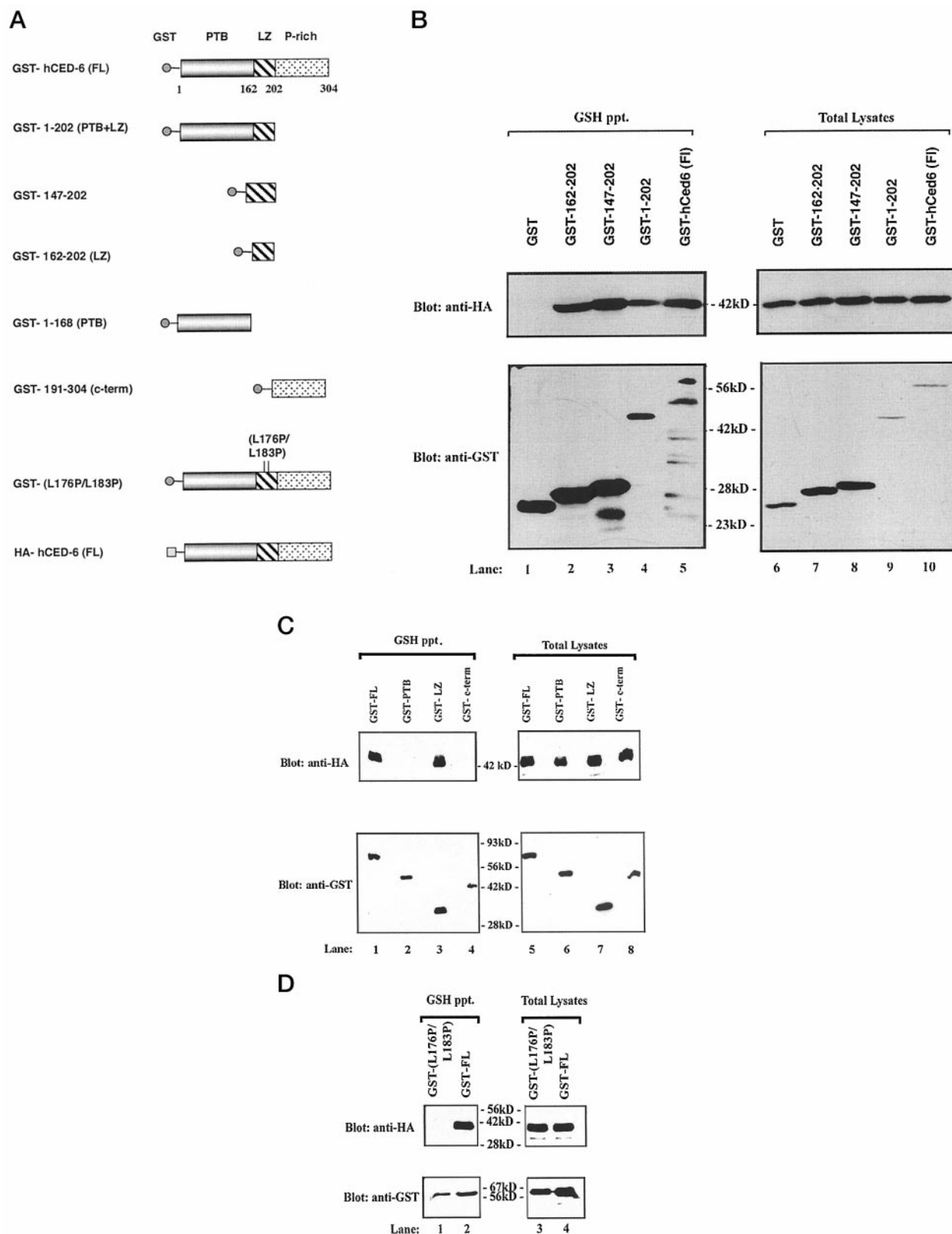


FIG. 3. Dimerization of hCED-6 is mediated through its leucine zipper domain (amino acids 162–202). *A*, schematic representation of the hCED-6 constructs generated with GST and HA tags. *B*, GST-tagged hCED-6 truncations containing the leucine zipper domain were co-transfected with HA-tagged full-length hCED-6. Following GST precipitation, the co-precipitated HA-hCED-6 proteins were revealed by anti-HA immunoblotting. Although the predicted dimerization region starts from amino acid 147, a construct starting from amino acid 162 (*lane 2*) appears to precipitate hCED-6 as efficiently as the construct starting from amino acid 147 (*lane 3*). Expression of all the transfected proteins was confirmed by immunoblotting of the total cell lysates (*lanes 6–10*). *C*, to test the ability of isolated GST-tagged PTB, LZ, and C terminus to dimerize with full-length HA-hCED-6, these constructs were transfected into COS cells, precipitated with GSH beads, and analyzed by anti-HA immunoblotting (*upper panels*). The levels of GST-tagged proteins were determined by anti-GST immunoblotting (*lower panels*). Comparable expression of all transfected proteins in total cell lysates is also shown (*lanes 5–8*). *D*, the central two leucines within the leucine zipper domain were mutated to prolines in full-length hCED-6. GST-tagged (L176/L183P) hCED-6 was co-transfected with HA-hCED-6 and analyzed as described in the legend to Fig. 1. Comparable levels of the transfected proteins were expressed in total cell lysates (*lanes 3 and 4*).

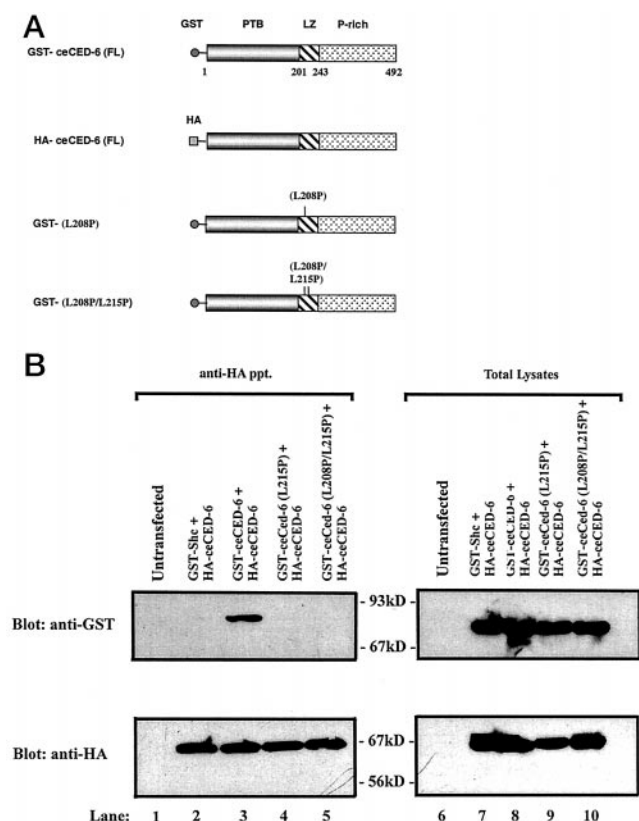


FIG. 4. ceCED-6 also dimerizes through a leucine zipper domain. *A*, schematic diagrams of the GST and HA-tagged ceCED-6 constructs and the mutations within the leucine zipper. *B*, the different GST-tagged constructs were co-expressed with HA-ceCED-6 in 293T cells. Following anti-HA immunoprecipitation co-precipitated GST-tagged proteins were detected by anti-GST immunoblotting. GST-ceCED-6 can co-precipitate with HA-tagged ceCED-6 (lane 3). When a single leucine is mutated to proline, the dimerization was abrogated (lane 4). The levels of HA-ceCED-6 precipitated in the different lanes are shown in the *bottom panel* (left). The expression of all the GST-tagged proteins and HA-tagged proteins in total lysates are shown in the *upper right* and *lower right panels*, respectively (lanes 6–10).

These data demonstrate that the ceCED-6 protein also dimerizes through the LZ domain.

Species-specific Dimerization of hCED-6 and ceCED-6—Since it appeared that the LZ-dependent dimerization of CED-6 is evolutionarily conserved, we tested whether hCED-6 would be capable of dimerizing with ceCED-6. To explore this possibility, we co-expressed either HA-tagged or GST-tagged hCED-6 with the oppositely tagged GST or HA-ceCED-6 in 293T cells. The lysates were precipitated with anti-HA antibodies, and the co-precipitation of the GST-tagged protein was determined by anti-GST immunoblotting. As shown in Fig. 5, HA-tagged ceCED-6 was unable to cross-dimerize with GST-hCED-6 but was fully capable of dimerizing with GST-tagged ceCED-6. Likewise, hCED-6, although capable of dimerizing to itself, was unable to cross-dimerize with ceCED-6. These results underscore the high specificity for leucine zipper-dependent interactions in CED-6. In some experiments, after long exposure, we detected bands corresponding to ceCED-6 co-precipitating with hCED-6. Because these were extremely weak compared with the strong bands observed with CED-6 from the same species, we conclude that the cross-species interaction is not strong.

Evidence for Dimerization of Rodent CED-6—The presence of a conserved leucine zipper domain from worms to humans suggested an evolutionarily important role for this domain in CED-6 function that may also be conserved in other species.

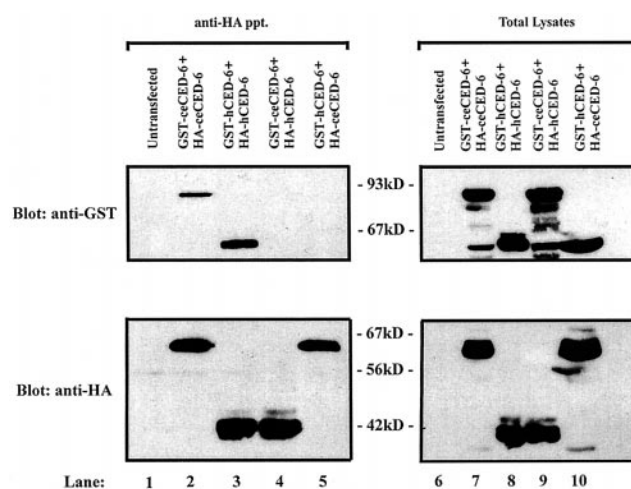


FIG. 5. hCED-6 does not cross-dimerize with ceCED-6. GST-tagged hCED-6 and ceCED-6 were co-expressed with HA-tagged hCED-6 and ceCED-6. Following anti-HA immunoprecipitation co-precipitated GST-tagged proteins were detected with anti-GST immunoblotting. GST-ceCED-6 was only co-precipitated by HA-ceCED-6 (lane 2). Likewise, GST-hCED-6 was only co-precipitated by HA-hCED-6 (lane 3). Comparable HA-CED-6 was precipitated in all lanes (*left bottom panel*). Expression of the transfected proteins in total lysates is shown in lanes 7–10 (*right panels*).

Thus, we predicted that a similar dimerization also occurs in the rodent CED-6 protein. We have observed that the anti-human CED-6 antibodies (generated against a peptide sequence derived from the hCED-6 PTB domain) also recognized a protein in the hamster LR73 cell line with a molecular weight similar to that of the human protein (data not shown). It is noteworthy that the anti-hCED-6 antibodies do not cross-react with the ceCED-6 (data not shown). Based on this, we hypothesized that the rodent and human proteins are more closely related and that they may be able to cross-dimerize. This would also reveal the presence of a similar LZ domain, even though the hamster *ced-6* gene has not yet been cloned. To test this possibility, we stably expressed GST-hCED-6 in LR73 cells. These cells were lysed and precipitated with GSH beads, and the co-precipitation of the endogenous hamster CED-6 was analyzed by anti-CED-6 immunoblotting. As shown in Fig. 6A, hamster CED-6 was found to co-precipitate with the GST-hCED-6 from transfected cells but was not precipitated from the parental LR73 cells. Expression of GST-LZ alone of hCED-6 in LR73 cells also co-precipitated the endogenous hamster CED-6 (data not shown).

To further characterize the oligomeric state of endogenous hamster CED-6, lysates from parental untransfected LR73 cells were fractionated by size exclusion chromatography. The fractions were separated by SDS-PAGE, and the presence of hamster CED-6 in the individual fractions was analyzed by anti-hCED-6 immunoblotting. If endogenous CED-6 in LR73 cells were to exist as a dimer *in vivo* and assuming a globular shape, this CED-6 protein would be expected to have a similar elution pattern as proteins of ~70 kDa. As seen in Fig. 6B, the peak of hamster CED-6 was observed between fractions 12 and 13. In comparison, the adapter protein Grb2 (predicted molecular mass of 23 kDa) appears in fraction 20. In the same experiment, the peak of the different isoforms of Shc, p66, p52, and p48 were seen in fractions 13, 14/15, and 15, respectively. From these data, we conclude that the endogenous hamster CED-6 migrates closer to proteins of 65–70 kDa (rather than as the predicted 35-kDa monomeric form) and also exists as a dimer *in vivo*.

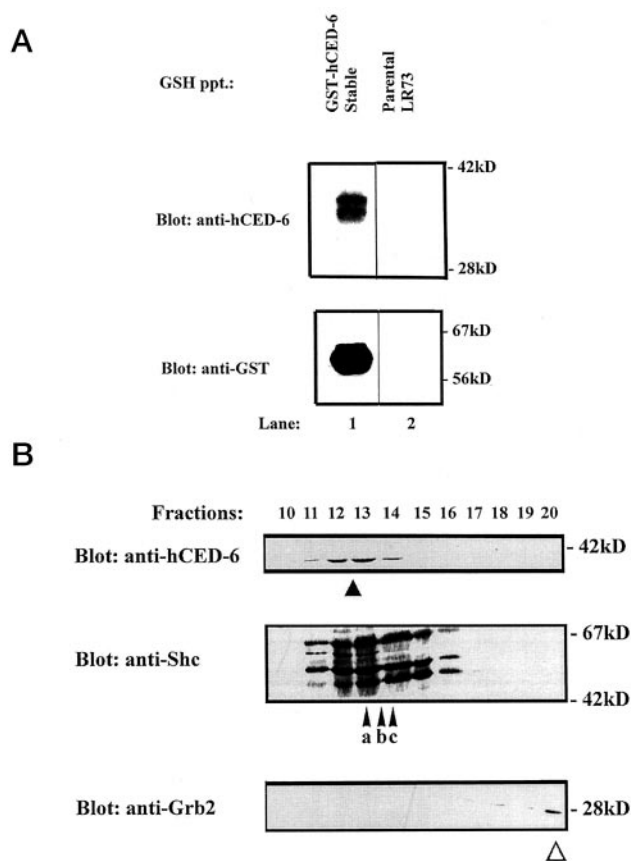


FIG. 6. Rodent CED-6 also can form dimers. A, Chinese hamster ovarian fibroblast cell line LR73 was stably transfected with GST-tagged hCED-6. Lysates from parental LR73 cells or the GST-hCED-6 transfected cells were precipitated with GSH beads and the co-precipitated hamster CED-6 was detected with antibody to hCED-6, which cross-reacts with the hamster CED-6 protein. The expression of GST-hCED-6 is shown in the bottom panel. B, to characterize the oligomeric state of endogenous CED-6 protein, parental LR73 cells were lysed, and the proteins were separated by size exclusion chromatography as described under "Experimental Procedures." The fractions were run by SDS-PAGE, and the presence of CED-6, as well as Shc and Grb2 proteins, were determined by immunoblotting (top, middle, and lower panels, respectively). Arrows at the bottom indicate the apparent peak of proteins in the different fractions. The peak of CED-6 was noted between fractions 12 and 13 (solid triangle), whereas Shc isoforms, p66, p52, and p46 eluted with peaks between fractions 13, 14/15, and 15 (a, b, and c, respectively). The peak of Grb2 elution was in fraction 20 (open triangle). The hamster CED-6 elution profile was more characteristic of a protein of ~70 kDa (suggestive of a dimer) rather than around 35 kDa as expected of monomeric CED-6.

DISCUSSION

In this report, we demonstrate that CED-6, an adapter protein involved in phagocytosis of apoptotic cells, can form homodimers *in vivo*. This CED-6 leucine zipper is necessary and sufficient for dimerization between CED-6 proteins and is evolutionarily conserved from *C. elegans* to man. Our data suggest that CED-6 protein in rodents, although not yet cloned, also exists as a dimer *in vivo* and may contain a similar LZ domain involved in dimerization. To our knowledge, CED-6 is the only known PTB domain with a contiguous leucine zipper dimerization domain and suggests another possible mode of regulation for ligand binding through the PTB domains. Because the ligands for CED-6 are currently unknown and the signaling events involved in phagocytosis of apoptotic cells are just beginning to be elucidated, the dimerization demonstrated here has implications for regulation of engulfment by CED-6.

The Multicoil program was designed to identify parallel coiled-coil structures and furthermore distinguish between po-

tential dimers and trimers (20). The predictions are based on the location of certain types of amino acids in heptad repeats, with positions within the repeat being denoted as (abcdefg)_n. The positions of characteristic hydrophobic residues in positions a and d and charged residues in positions e and g within the heptad determine the coiled-coil. Moreover, Multicoil considers the location and the pairwise interactions between the residues within the heptad to distinguish a given coiled-coil as a dimer or trimer (20). Based on this, the coiled-coil within both the hCED-6 and ceCED-6 proteins were classified as dimers. Although the shoulders of the coiled-coil regions of both human and *C. elegans* proteins were assigned some probability score for being a trimer, this is most likely an artifact of the algorithm in predicting multimerization at the shoulders of coiled-coils.² Moreover, the smaller "bumps" on the Multicoil prediction of the ceCED-6 around amino acids 100 and 450 are not considered significant because of the low probability scores. In addition, our mutational analyses of human and ceCED-6 demonstrated that the CED-6 dimerization is dependent on the leucine zipper motif.

The helical wheel prediction provides further evidence for dimerization of the coiled-coil region of CED-6. The leucines and the hydrophobic residues are well aligned on one side of the coiled-coil, whereas the hydrophilic residues are distributed evenly throughout the rest of the coiled-coil. This again favors the formation of a dimer. Moreover, our size exclusion studies point to CED-6 being a dimer. Despite the caveats of size exclusion chromatography such as the globular *versus* elongated shape of the molecule, hamster CED-6 was present in fractions earlier than those in which p66 and p52 Shc proteins were seen. The Shc isoforms have a PTB domain and a proline-rich region analogous to CED-6 but do not have a leucine zipper region; our previous studies have also demonstrated that Shc exists only as a monomer (17). Taken together, we conclude that CED-6 proteins exist as dimers *in vivo*. However, we cannot completely rule out the possibility that higher order oligomers can form, possibly building on the basic block of the dimer. Since the gel filtration studies suggested that the majority of CED-6 existed in a homodimeric state, any hamster CED-6 that forms higher order oligomers would constitute a small fraction of the total population of hamster CED-6 that is too small to detect by immunoblotting. An interesting possibility is that higher order oligomers of rodent CED-6 may form during an activation event such as recognition of apoptotic cells or initiation of phagocytosis. It is noteworthy that although our data support a role for the leucine zipper of CED-6 in homodimerization, the results do not exclude heterodimerization with other LZ-containing proteins. The above possibilities are currently under investigation.

Our data also demonstrate that the leucine zipper domain is necessary and sufficient for dimerization of CED-6 proteins. The isolated PTB domain or the C-terminal region were unable to co-precipitate full-length hCED-6, whereas the LZ alone was very efficient in co-precipitating full-length CED-6. In this regard, we observed that the quantity of HA-CED-6 precipitated by GST-tagged full-length CED-6 or GST-LZ was indistinguishable in many different experiments. Although we cannot rule out small contribution from other regions of CED-6 to dimerization of the native protein, these observations and our data that point mutations of the critical leucines in the LZ region abolishes the dimerization leads us to propose that the LZ domain is the primary, if not the only region involved in dimerization of CED-6. The interactions via the LZ of CED-6 also appeared quite specific. The failure of human or rodent

² P. Kim, personal communication.

CED-6 protein to dimerize with the worm protein or other PTB domain-containing proteins such as Shc supports this conclusion. We also observe that stable expression of ceCED-6 in LR73 cells, unlike human CED-6, fails to co-precipitate the endogenous hamster CED-6.³

The leucine zipper identified in the human and *C. elegans* CED-6 proteins and the data with endogenous hamster proteins suggest that the dimerization domain is evolutionarily conserved and is likely to be important for CED-6 function. Genetic studies strongly implied that CED-6 functions as a signaling adapter protein during engulfment of apoptotic cells (10). Expression of human *ced-6* transgene in worms carrying mutations of *ced-6* partially rescued the defect in clearance of apoptotic bodies, suggesting that the mechanism of CED-6 function is conserved through evolution (15). Consistent with this, the overall structural features of CED-6 are highly conserved in the different species with a PTB domain at its N terminus, followed by the LZ domain and then the C-terminal proline-rich domain. Although ligands for CED-6 remain to be identified, each of its three modules could potentially mediate interactions with ligands. Based on the known function of other PTB domains in binding to surface receptors, it is believed that the PTB domain would mediate interactions with surface receptors involved in phagocytosis, whereas the C-terminal proline-rich region would interact with intracellular signaling proteins and possibly regulate cytoskeletal reorganization during engulfment. We favor a model in which the PTB and C-terminal regions interact with ligands, whereas the leucine zipper mediates homodimerization of CED-6. The LZ-dependent dimerization may aid the binding of CED-6 to its ligands. For example, the ligand for the PTB domain may contain more than one PTB domain-binding site, and dimerization may facilitate CED-6 binding to such ligands. Alternatively, the affinity of the PTB domain for its ligand may be low, and dimerization may enhance such low affinity interactions. PTB domains have structural homology to pleckstrin homology domains and PTB domains of Shc and Dab-1 have been shown to bind phospholipids (21, 22). Since the affinity of PTB domains for phospholipids is lower than that for protein ligands (22, 23), the phospholipid interactions would be aided by binding through dimerized PTB domains.

The dimerization of CED-6 may also improve its ability to bind to membrane proteins. Since many membrane receptors function through dimerization, it is possible that CED-6 binds to membrane receptors involved in phagocytosis after they dimerize. Alternatively, CED-6 may help to cluster receptors involved in phagocytosis, and this could be part of the mechanism of engulfment. Some of the surface receptors implicated in phagocytosis of apoptotic cells can form multimers (e.g. scav-

enger receptors) (24, 25), and further clustering of these already oligomerized receptors through CED-6 may be a mechanism involved in phagocytosis. Additionally, the C-terminal domain of CED-6 contains potential SH3-binding sites. SH3 interactions are generally low affinity interactions, and dimerization of CED-6 could allow proteins, which contain multiple SH3 domains, for example Grb2 and Crk, to bind with higher affinity. It is interesting to note that the large adapter protein Cbl, which contains a domain that interacts with phosphotyrosines, has been shown to dimerize through a leucine zipper domain, and this dimerization appears to be required for phosphorylation of Cbl (26). Elucidating the precise role of CED-6 dimerization in signaling during phagocytosis of apoptotic cells may provide important insights to our molecular understanding of this critical biological process.

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