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General interaction mode of CIDE:CIDE complex revealed by a mutation study of the Drep2 CIDE domain

Seung Mi Lee, Hyun Ho Park *

School of Biotechnology and Graduate School of Biochemistry, Yeungnam University, Gyeongsan, Republic of Korea

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ABSTRACT

The CIDE domain is a well known protein–protein interaction module that is initially detected at the apoptotic DNA fragmentation factor (DFF40/45). The interaction mechanism via the CIDE domain is not well understood. To elucidate CIDE domain mediated interactions in the apoptotic DNA fragmentation system, we conducted biochemical and mutational studies and found that the surface of CIDE domains can be divided into an acidic side and a basic side. In addition, a mutagenesis study revealed that the basic surface side of Drep2 CIDE is involved in the interaction with the acidic surface side of Drep1 CIDE and Drep3 CIDE. Our research supports the idea that a charge–charge interaction might be the general interaction mode of the CIDE:CIDE interaction.

Structured summary of protein interactions:
Drep2 and **Drep2** bind by molecular sieving (View Interaction: 1, 2)
Drep1 and **Drep2** bind by molecular sieving (View interaction)
Drep3 and **Drep2** bind by molecular sieving (View interaction)
Drep2 and **Drep3** bind by blue native page (View interaction)
Drep2 and **Drep1** bind by blue native page (View interaction)

1. Introduction

DNA fragmentation is a well known biochemical feature of apoptosis [1–3], and it is well known that DNA fragmentation factor 40/45 (DFF40/45), apoptosis inducing factor (AIF), and EndoG are involved in this apoptotic DNA fragmentation. Previous studies showed that there are two distinct stages of DNA fragmentation during the apoptosis process, large-scale DNA fragmentation and further cleavage [1,4]. The initial large-scale DNA fragmentation is mediated by AIF, which is located in the mitochondria under normal conditions but released by apoptotic stimuli [5]. Further small-scale DNA cleavage is primarily mediated by a DFF40/45 hetero-dimeric complex [2]. During this process, the DNA undergoes fragmentation and is eventually cleaved into regular ~180 bp fragments [2,3]. DFF40 is a novel nuclease that is involved in the direct DNA cleavage process, while DFF45 is an inhibitor that can suppress the nuclease activity of DFF40 under normal conditions via a tight interaction [3,6]. Interestingly, DFF45 also has chaperon activity for DFF40 during its synthesis [7]. DFF45 is cleaved by effector caspases such as caspase-3 during the apoptosis process, allowing DFF40 to be dissociated from DFF45, enter the nucleus, and degrade chromosomal DNA [3,8,9].

Apoptotic DNA fragmentation is conserved in fly, and several homologous proteins have been identified by sequence analysis [10]. DFF-like proteins possess one or two CIDE domains for the protein:protein interaction and the presence of the CIDE domain in the proteins is an indicator of DFF-like protein [11]. Based on this approach, Drep1, Drep2, Drep3, and Drep4 have been identified as DFF-like proteins in fly [10]. All four proteins have a conserved CIDE domain that mediates protein–protein interactions. An initial study showed that Drep1 and Drep4 are DFF45 and DFF40 homologues, respectively [12,13]. Although the function of Drep2 and Drep3 are still unclear, a recent study showed that Drep2 might be another apoptotic nuclease that is inhibited by Drep3 by a tight interaction via the CIDE domain [14]. Additionally, a biochemical study recently revealed that Drep2 interacts with Drep1 via the CIDE domain [15]. However, the biological implications of the interaction between Drep2 and Drep1 have yet to be elucidated. Overall, recent studies have shown that Drep2 interacts with Drep3, a Drep2 inhibitor, and Drep1, a Drep4 inhibitor. All interactions are mediated by CIDE domains.

To elucidate CIDE domain mediated interactions in the apoptotic DNA fragmentation system, we performed biochemical and mutational studies using purified CIDE domains of Drep1, Drep2, and Drep3. In this study, we speculated the amino acid residues

* Corresponding author. Address: Department of Biotechnology, School of Biotechnology and Graduate School of Biochemistry, Yeungnam University, Gyeongsan, Republic of Korea. Fax: +82 053 810 4769.
E-mail address: hyunho@ynu.ac.kr (H.H. Park).

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on the Drep2 CIDE which might be critical for interaction with Drep1 and Drep3 based on sequence alignment and structural comparison with previously solved complex structures between DFF40 CIDE (CAD CIDE) and DFF45 CIDE (ICAD CIDE) [11,16]. Moreover, the mutagenesis study revealed that the basic surface side of Drep2 CIDE is involved in the interaction with the acidic surface side of Drep1 CIDE and Drep3 CIDE. In addition, we found that K40, not R36, on the basic surface side of Drep2 CIDE is critical to the interaction with Drep1 CIDE and Drep3 CIDE. Overall, the results of this study indicate that interactions are mediated by different charged surfaces, and this might be the general case for CIDE:CIDE interactions.

2. Materials and methods

2.1. Homology modeling of the structures of the CIDE domain

Homology models of Drep1 CIDE, Drep2 CIDE, and Drep3 CIDE were constructed using a homology modeling server, SWISS-MODEL [17]. The DFF45 structure (PDBid: 1IBX) for Drep1 CIDE, CIDE-A structure (PDBid: 2EEL) for Drep2 CIDE, and CIDE B structure (PDBid: 1D4B) for Drep3 CIDE were used as the modeling templates. The stereochemical quality of constructed models was validated using a Ramachandran plot generated with PROCHECK [18]. Electrostatic surfaces and ribbon diagrams were generated using the PyMOL program (DeLano, W. L. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, San Carlos).

2.2. Mutagenesis

Site-directed mutagenesis was conducted using a Quick-change kit (Stratagene) according to the manufacturer's protocols. Mutagenesis was then confirmed by sequencing. Mutant proteins were prepared using the same method described above.

2.3. Target protein expression and purification

Expression and purification of Drep1 CIDE (amino acid residues: 1–90), Drep2 CIDE (amino acid residues: 1–84), and Drep3 CIDE (amino acid residues: 111–195) were conducted as in previous studies [14,19]. Briefly, Drep1 CIDE was cloned into pET28a vector, Drep2 was cloned into pET26a vector, and Drep3 CIDE was cloned into homemade pOKD vector [20]. The plasmids containing the CIDE domains of all three proteins were then transformed into BL21 (DE3) *Escherichia coli* competent cells, after which expression was induced by treating the bacteria with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 293 K. The bacteria were then collected, resuspended and lysed by sonication in 50 ml lysis buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 10 mM imidazole). Next, the bacterial lysate was centrifuged at 16 000 rpm for 1 h at 4 °C. The supernatant fraction was subsequently applied to a gravity-flow column (Bio-Rad) packed with Ni–NTA affinity resin (Qiagen). The unbound bacterial proteins were removed from the column using washing buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 60 mM imidazole and 10% glycerol), after which the target proteins were eluted using an elution buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, and 250 mM imidazole). The protein purity was further improved using a Superdex 200 gel-filtration column (GE Healthcare). Drep2 CIDE mutants (Drep2R36E and Drep2K40E) were expressed and purified using a method similar to that used for purification the other CIDE domains.

2.4. Complex assay using gel-filtration chromatography

For gel filtration analysis to detect complex formation, each purified target proteins was mixed with each other and applied to a gel-filtration column (Superdex 200 HR 10/30, GE healthcare) that was pre-equilibrated with 20 mM Tris–HCl 8.0 and 150 mM NaCl. The peak fractions were collected and subjected to SDS–PAGE.

2.5. Native PAGE shift assay

The protein interaction between CIDE domains was monitored by native (non-denaturing) PAGE on a PhastSystem (GE Healthcare) with pre-made 8–25% acrylamide gradient gels (GE Healthcare). Separately purified proteins were pre-incubated at room temperature for 1 h before loading the gel. Coomassie Brilliant Blue was used for staining and detecting shifted bands.

3. Results and discussion

3.1. The surface of CIDE domains can be divided into two distinctly different charged sides, which might be critical for the interaction with their partners

DNA fragmentation, which is mediated by the DFF40/DFF45 heterocomplex, is the hallmark of apoptotic cell death and conserved among different species including *fly*. Four DFF-like proteins have been identified in *fly*, Drep1 (dDFF45), Drep2, Drep3 and Drep4 (dDFF40). Although the functional relationship between Dreps is not clear, a recent study showed that Drep2 is a novel nuclease that can interact with both Drep3 and Drep1 via the CIDE domain [14]. CIDE domain-mediated DFF40 and DFF45 interaction is critical to regulation of the nuclease activity of DFF40.

Sequence alignment was conducted to determine whether CIDE domains of Dreps in *fly* use similar modes of interactions as the DFF40:DFF45 system. The alignment results showed that the CIDE domain was conserved at Drep1, Drep2, Drep3, and Drep4. The important residues for the interaction between DFF40 CIDE and DFF45 CIDE were K9, K18, K32, and R36 on DFF40 CIDE and D66, D71, D72 and D74 on DFF45 CIDE, and those residues were conserved on the CIDE of Dreps [21] (Fig. 1A). Interestingly, the Drep CIDE domains contain both conserved acidic and basic residues, unlike DFF40 and DFF45, which contain only conserved basic residues on DFF40 and conserved acidic residues on DFF45 (Fig. 1A). A previous structural study showed that the interaction between DFF40 CIDE and DFF45 CIDE was largely mediated by charge–charge interaction (Fig. 1B) [21]. We also conducted homology modeling using the DFF45 structure (PDBid: 1IBX) for Drep1 CIDE, the CIDE-A structure (PDBid: 2EEL) for Drep2 CIDE, and the CIDE-B structure (PDBid: 1D4B) for Drep3 CIDE as a modeling template and the surface charges of each CIDE were calculated (Fig. 1C) [11,21]. The gross features of each electrostatic surface were very similar to those of DFF40 CIDE and DFF45 CIDE in that one side of CIDE is acidic and another side is basic (Fig. 1C). Because CIDEs are protein interaction modules, their surface features indicate their modes of interactions with partners. Although no structural information is available, the sequence alignment and modeling studies suggested that the conserved charged surfaces of CIDEs are important for their interactions with partners.

3.2. Basic-charged side of Drep2 CIDE is involved in the interaction with Drep1 CIDE and Drep3 CIDE

The surface features of Drep2 CIDE are also divided into two distinctly different charged sides, an acidic side and a basic side (Fig. 1D). Previous study indicated that Drep2 CIDE interact to Drep3 CIDE [14] and the most recent study showed that Drep2 CIDE might interact directly to Drep1 CIDE [15]. However, it is not known which side of Drep2 CIDE interacts with which side of Drep1 CIDE and Drep3 CIDE. As a first step in elucidation of the

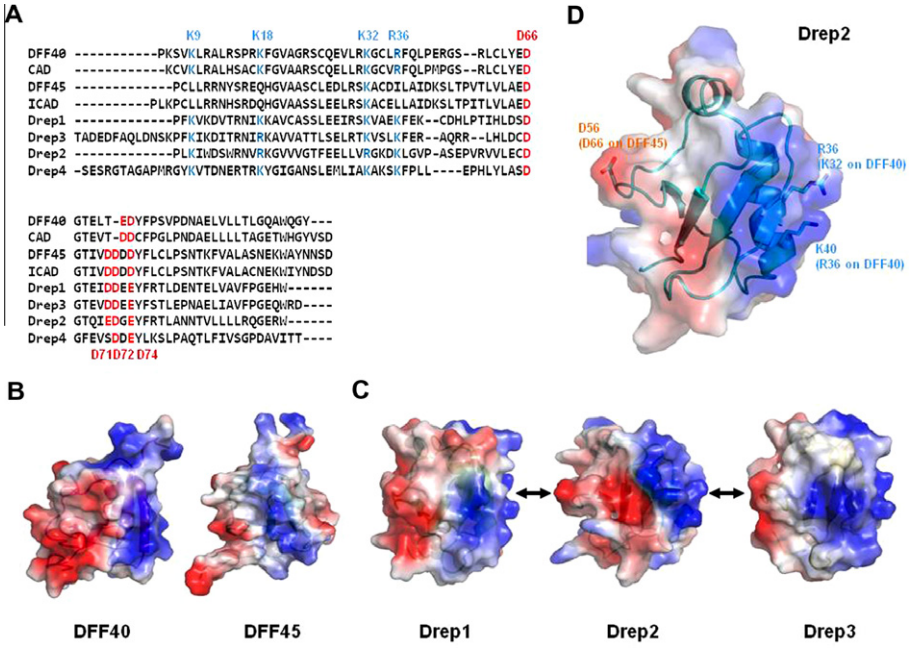

Fig. 1. Charged surface-mediated CIDE–IDE interaction might be universal across species. (A) Sequence alignment of CIDE domains. Residues conserved and involved in the interaction between DFF40 (CAD) and DFF45 (ICAD) are colored at blue for basic residues and red for acidic residues. (B) Structure of DFF40 CIDE:DFF45 CIDE complex. The electrostatic surface is shown. The basic-charged surface of DFF40 interacts with the acidic-charged surface of DFF45. Electrostatic surfaces were calculated by PyMOL. (C) Electrostatic surface representations of the homology modeled CIDE domain of Drep1, Drep2, and Drep3 are shown. Arrows indicate previously identified interactions between Dreps. (D) Homology model of Drep2. Electrostatic surfaces are shown. Conserved residues that might be important to the interaction with its binding partners are presented. R36 and K40 on Drep2 CIDE correspond to K32 and R36 on DFF40 based on the sequence alignment. D56 on Drep2 CIDE corresponds to D66 on DFF45 based on the sequence alignment.

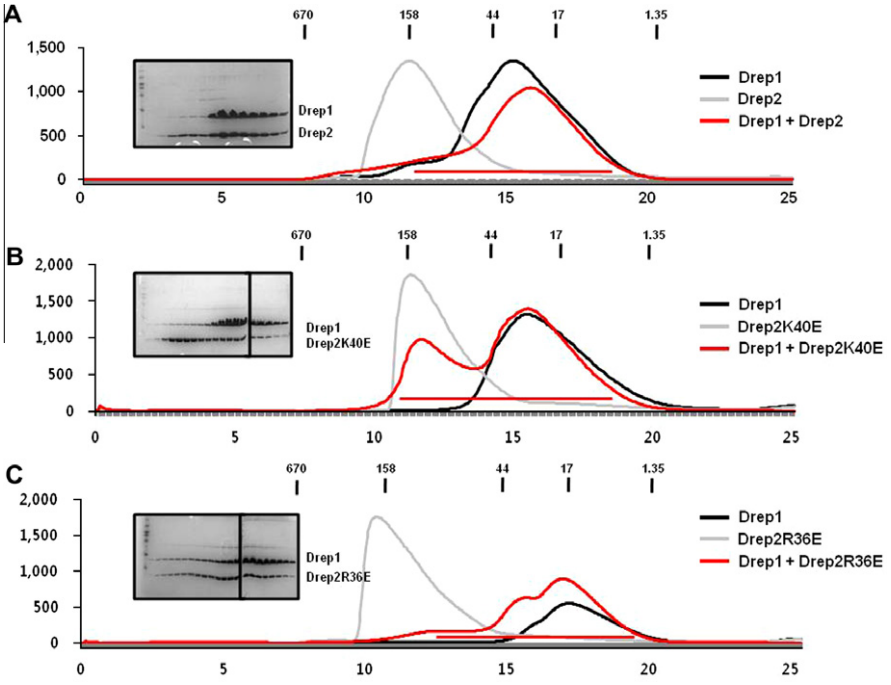

Fig. 2. Basic surface disruption mutant, Drep2K40E, did not bind to Drep1. (A) Gel-filtration chromatograms and fractions of Drep1 CIDE, Drep2 CIDE, and mixture of Drep1 CIDE and Drep2 CIDE. The profile obtained from Drep1 CIDE is indicated by the black line, Drep2 CIDE by the gray line, and the mixture by the red line. All gel-filtration chromatography was performed using 20 mM Tris–HCl and 150 mM NaCl buffer. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep1 CIDE and Drep2 CIDE are shown on the left side. (B) Gel-filtration chromatograms and fractions of Drep1 CIDE, Drep2K40E, and the mixture of Drep1 CIDE and Drep2K40E CIDE. The profile obtained from Drep1 CIDE is indicated by the black line, Drep2K40E by the gray line, and the mixture by the red line. All gel-filtration chromatography was performed using 20 mM Tris–HCl and 150 mM NaCl buffer. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep1 CIDE and Drep2K40E are shown on the left side. (C) Gel-filtration chromatograms and fractions of Drep1 CIDE, Drep2R36E, and the mixture of Drep1 CIDE and Drep2R36E. The profile obtained from Drep1 CIDE is indicated by the black line, Drep3R36E by the gray line, and the complex by the red line. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep1 CIDE and Drep2R36E are shown on the left side.

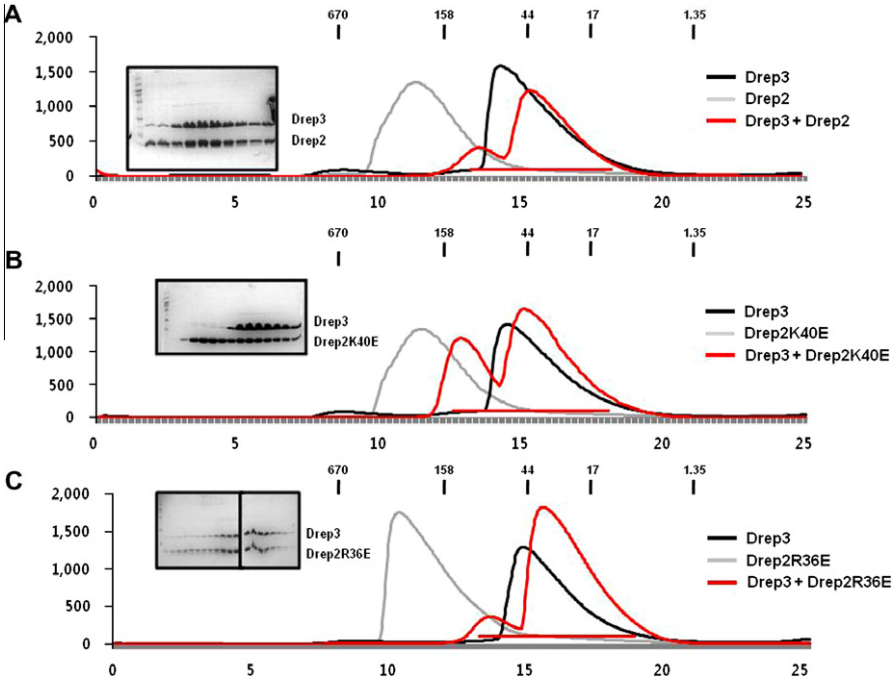

Fig. 3. Basic surface disruption mutant, Drep2K40E, did not bind to Drep3. (A) Gel-filtration chromatograms and fractions of Drep3 CIDE, Drep2 CIDE, and mixture of Drep3 CIDE and Drep2 CIDE. The profile obtained from Drep3 CIDE is indicated by the black line, Drep2 CIDE by the gray line, and the mixture by the red line. All gel-filtration chromatography was performed using 20 mM Tris–HCl and 150 mM NaCl buffer. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep3 CIDE and Drep2 CIDE are shown on the left side. (B) Gel-filtration chromatograms and fractions of Drep3 CIDE, Drep2K40E, and the mixture of Drep3 CIDE and Drep2K40E CIDE. The profile obtained from Drep3 CIDE is indicated by the black line, Drep2K40E by the gray line, and the mixture by the red line. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep3 CIDE and Drep2K40E are shown on the left side. (C) Gel-filtration chromatograms and fractions of Drep3 CIDE, Drep2R36E, and the mixture of Drep3 CIDE and Drep2R36E. The profile obtained from Drep3 CIDE is indicated by the black line, Drep3R36E by the gray line, and the complex by the red line. SDS–PAGE loaded fractions (red-bar) of gel-filtration chromatography obtained from the mixture of Drep3 CIDE and Drep2R36E are shown on the left side.

interaction mode of Drep2 CIDE, we purified each CIDE domain of Drep1, Drep2, and Drep3 and generated mutants of Drep2 CIDE. While R36 and K40, which might be important to the interaction on the basic side, were mutated to oppositely charged glutamic acid (Drep2R36E and Drep2K40E), D56 on the opposite acidic side was mutated to arginine (Drep2D56R). Although Drep1 CIDE, Drep2 CIDE, Drep3 CIDE, Drep2R36E and Drep2K40E were expressed well, Drep2D56R was not expressed properly. Due to the unavailability of Drep2D56R, we decided to conduct further experiments using two mutants, Drep2R36E and Drep2K40E, to analyze the interactions.

Drep2 CIDE forms a highly oligomeric self complex in solution that elutes from the gel-filtration column at around 12 ml (Fig. 2A) unlike Drep1CIDE and Drep3 CIDE (data not shown). Drep2 CIDE forms an octamer or decamer as the calculated molecular weight of monomeric Drep2 CIDE is 11,324 Da. Once Drep2 CIDE formed a hetero-complex with Drep1 CIDE, the Drep2 CIDE peak disappeared and a complex peak was detected at around 16 ml on the gel-filtration column (Fig. 2A). SDS–PAGE showing the co-migration of Drep2 CIDE and Drep1 CIDE indicates that Drep2 CIDE forms a stable complex with Drep1 CIDE. The interaction between Drep1 CIDE and Drep2 CIDE has previously been reported [15]. To analyze the interaction between Drep1 and Drep2K40E, and Drep1 and Drep2R36E, we mixed the two proteins and incubated them at room temperature for one hour, after which they were loaded onto the gel-filtration column. The profiles showed that Drep2K40E was not able to form a complex with Drep1 CIDE, as indicated by a Drep2 self-complex peak at around 12 ml (Fig. 2B). SDS–PAGE showing two proteins that migrated separately supports the data obtained from gel-filtration chromatography (Fig. 2B). However, Drep2R36E still interacts with Drep1 CIDE. The peak indicating self-complex of Drep2 CIDE was no longer present, and Drep1 CIDE was found to co-migrate with Drep2R36E upon SDS–PAGE (Fig. 2C). These results showed that Drep2 CIDE interacts with Drep1 CIDE using the basic side and K40, not R36, is the critical residue involved in the interaction.

After analyzing the interaction between Drep2 CIDE and Drep1 CIDE, we investigated whether Drep2 CIDE could interact with Drep3 CIDE using the same interaction mode with the same binding side. To accomplish this, we again conducted gel-filtration chromatography followed by SDS–PAGE and checked the position of the peak and co-migration. To analyze the interaction between Drep2 and Drep3, purified Drep2 CIDE and two mutants were mixed with Drep3 CIDE and then incubated for 1 h at room temperature, after which they were applied onto an S-200 gel-filtration column. A complex of Drep2 CIDE:Drep3 CIDE was obtained as shown by gel filtration chromatography and SDS–PAGE (Fig. 3A). The obviously disappearing self-complex peak around 12 ml and co-migration on the SDS–PAGE indicated that Drep2 CIDE interacts with Drep3 CIDE (Fig. 3A). Drep3 and Drep2K40E or Drep3 and Drep2R36E mixtures were also produced and loaded onto the gel-filtration column. The profiles and SDS–PAGE showed that Drep2K40E could not form a complex with Drep3 CIDE, but that Drep2R36E interacts with Drep3 CIDE (Fig. 3B and C). The Drep2 peak moved slightly when it was mixed with Drep2K40E, but the reason for this phenomenon is not clear.

The interactions were also confirmed using native PAGE (Fig. 4). Although the mixtures of Drep1 CIDE:Drep2R36E, Drep3 CIDE:-Drep2R36E, and Drep3:Drep2K40E produced complex bands, the mixture of Drep1:Drep2K40E did not (Fig. 4). This result supports the fact that K40E on the basic surface side of Drep2 is crucial to the interaction with Drep1. Although the mixture of Drep3:

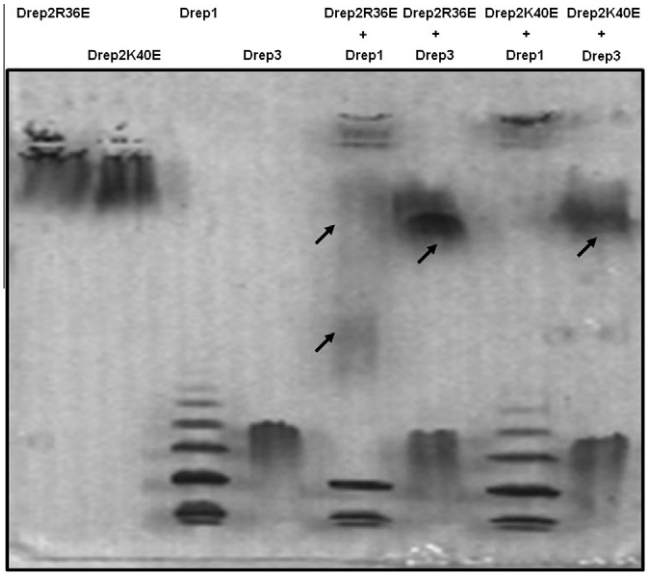

Fig. 4. Mutation effects are confirmed by native-PAGE. Loaded proteins on the native-PAGE are indicated. + indicates that two proteins were mixed. Newly formed complex bands are indicated by the arrows.

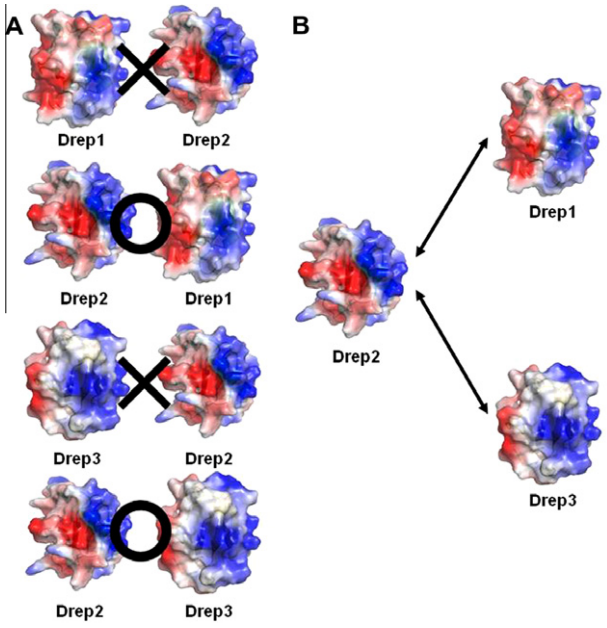

Fig. 5. Model of the interaction of Drep2 CIDE with Drep1 CIDE and Drep3 CIDE. (A) Correct orientations for the interaction of two CIDE proteins are marked with an O. Incorrect orientations that can be generated are marked with an X. (B) Final model of interaction between Drep2 CIDE and Drep1 CIDE, and between Drep2 CIDE and Drep3 CIDE.

Drep2K40E still produced complex bands, the amount of complex band was relatively small compared to the band generated by the mixture of Drep3:Drep2R36E, indicating that K40E is also important to binding with Drep3 CIDE. Previous gel-filtration and SDS–PAGE clearly showed that Drep2K40E was not able to form a complex with Drep3.

3.3. A predicted model of the interaction of Drep2 with its binding partners, Drep1 and Drep3

The interaction mode of the CIDE domain was not clearly characterized due to the lack of a structural study. Because of the insolubility and the tendency of not being crystallized, there is no available crystal structure of the CIDE domain currently available. However, our biochemical and mutational characterization of CIDE of Dreps indicates that CIDE domains have conserved charged residues on the surface that are critical to the interaction with binding partners. Interestingly, the surfaces of CIDE domains, including Drep1 CIDE, Drep2 CIDE, Drep3 CIDE, DFF40 CIDE, and DFF45 CIDE, are composed of two distinctly different charged sides, acidic and basic, and the interaction is mediated by the charged surfaces. Our study showed that two opposite charge-mediated interactions may be universal for the CIDE:CIDE interaction.

Based on the mutation study, Drep2 uses the basic charged side for interaction with Drep1 CIDE and Drep3 CIDE (Fig. 5A and B). Although there is a conserved acidic side on Drep2, which might be important to the interaction with other Dreps, the acidic side was not involved in the interaction with Drep1 CIDE and Drep3 CIDE. Both basic and acidic sides might be involved in the self-oligomerization of Drep2 CIDE. Further structural investigations are needed to determine why the acidic side of Drep2 CIDE cannot interact with basic side of Drep1 CIDE or Drep3 CIDE.

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