The PIDDosome, which is an oligomeric signaling complex composed of PIDD, RAIDD and caspase-2, can induce proximity-based dimerization and activation of caspase-2. In the PIDDosome assembly, the adaptor protein RAIDD interacts with PIDD and caspase-2 via CARD:CARD and DD:DD, respectively. To analyze the PIDDosome assembly, we purified all of the DD superfamily members and performed biochemical analyses. The results revealed that caspase-2 CARD is an insoluble protein that can be solubilized by its binding partner, RAIDD CARD, but not by full-length RAIDD; this indicates that full-length RAIDD in closed states cannot interact with caspase-2 CARD. Moreover, we found that caspase-2 CARD can be solubilized and interact with full-length RAIDD in the presence of PIDD DD, indicating that PIDD DD initially binds to RAIDD, after which caspase-2 can be recruited to RAIDD via a CARD:CARD interaction. Our study will be useful in determining the order of assembly of the PIDDosome. [BMB Reports 2013; 46(9): 471-476]
RAIDD and PIDD interact with each other via their DDs, while RAIDD and caspase-2 interact with each other via their CARDs (30, 31, 36, 37).

Previous structural and biochemical studies have shown that the core of the PIDDosome was composed of 5 PIDD DD and 7 RAIDD DD molecules with unique angles and the PIDDosome containing three protein components was successfully reconstituted in vitro (26,38). To further analyze the PIDDosome assembly, we purified all DD superfamily members that are critical to assembly of the PIDDosome. Although obtaining functional DD superfamily members has been difficult owing to insolubility of the DD superfamily under physiological conditions, we purified successfully all functional DD superfamily members, including caspase-2 CARD and RAIDD CARD, as well as the full-length RAIDD, RAIDD DD, and PIDD DD (39, 40). Our biochemical experiments revealed that caspase-2 CARD is an insoluble protein that can be solubilized by its binding partner, RAIDD CARD, but not by full-length RAIDD, indicating that full-length RAIDD in a closed state cannot interact with caspase-2 CARD. RAIDD has been reported to exist in both an opened and closed form, and it is not surprising that closed RAIDD cannot accommodate caspase-2 CARD. In addition, we found that caspase-2 CARD can be solubilized and can interact with full-length RAIDD in the presence of PIDD DD, indicating that PIDD DD initially binds to RAIDD, after which caspase-2 is recruited to RAIDD via CARD: CARD interaction. The results of the present study will be useful in determination of the order of assembly of the PIDDosome.

RESULTS AND DISCUSSION

Purification of DD superfamily members involved in assembly of the PIDDosome

The PIDDosome is a caspase-2 activating complex composed of 3 proteins: PIDD, RAIDD, and caspase-2. Caspase-2 is recruited by this complex, after which it is activated by a proximity induced self-cleavage mechanism. Complex formation is a critical step involved in the activation of caspase-2. The PIDDosome is assembled via a CARD: CARD interaction between RAIDD and caspase-2 and a DD: DD interaction between RAIDD and PIDD (Fig. 1A). Although recent studies have revealed important biochemical features of the PIDDosome, its assembly mechanism is still unclear.

To further understand the assembly mechanism involved in formation of the PIDDosome, especially the order of complex formation, we attempted to purify all of the DD superfamily members in the PIDDosome and generate stable complexes of them in vitro. Unlike other DD superfamily members that are soluble after overexpression in bacteria (Fig. 1B-E), caspase-2 CARD is insoluble when it is over-expressed (Fig. 1F). To overcome the insolubility of caspase-2 CARD and obtain the RAIDD and caspase-2 complex, we co-expressed RAIDD CARD with its binding partner, caspase-2 CARD, which did not contain any tag. As shown in Fig. 2A, caspase-2 was co-expressed, solubilized, and co-migrated with RAIDD CARD on SDS-PAGE. However, co-eluted caspase-2 CARD was soon aggregated and dissociated from RAIDD CARD. The gel-filtration profile showed that caspase-2 CARD was missing during the gel-filtration chromatography step (Fig. 2B). Based on these results, we concluded that caspase-2 CARD is an unstable member of the DD superfamily that is solubilized by its binding partner, RAIDD CARD. Although caspase-2 CARD can bind to RAIDD CARD in vitro, the binding is not complete and caspase-2 CARD remains unstable.

Caspase-2 CARD can be solubilized by its binding partner, RAIDD CARD, but not by full-length RAIDD

To analyze the interaction and solubilization of caspase-2 by
Fig. 2. Caspase-2 CARD can be solubilized by its binding partner, RAIDD CARD, but not by full-length RAIDD. (A) His-tag pull-down assay of caspase-2 CARD with RAIDD CARD. RAIDD CARD with His-tag and caspase-2 CARD without His-tag were co-expressed. Co-eluted caspase-2 CARD is shown. Lane 1, marker; Lane 2, supernatant of the cell lysate; Lane 3, pellet of the cell lysate; Lane 4, flow through after incubation with Ni-NTA beads; Lanes 5 and 6, washing; Lanes 7-8, imidazole eluted fraction. (B) Fractions from a pull-down assay containing RAIDD CARD and co-eluted caspase-2 CARD were subjected to gel filtration chromatography. The peak fractions (black bar) were loaded onto SDS-PAGE. (C) His-tag pull-down assay for caspase-2 CARD with full-length RAIDD. Full-length RAIDD with His-tag and caspase-2 CARD without His-tag were co-expressed. Lane 1, marker; Lane 2, supernatant of the cell lysate; Lane 3, pellet of the cell lysate; Lane 4, flow through after incubation with Ni-NTA beads; Lanes 5 and 6, washing; Lanes 7-8, imidazole eluted fractions. (D) Fractions from pull-down assay containing full-length RAIDD and co-eluted caspase-2 CARD were subjected to gel filtration chromatography. The peak fractions (black bar) were subjected to SDS-PAGE.

RAIDD, we conducted a pull-down assay with full-length RAIDD. RAIDD is a dynamic adaptor protein with a structure and solubility that is changed by pH and temperature (38); therefore, we did not expect full-length RAIDD to interact with caspase-2 CARD, even though separately purified RAIDD CARD and RAIDD DD can interact with caspase-2 CARD and PIDD DD, respectively. As expected, caspase-2 CARD without His-tag was not co-eluted with His-tagged full-length RAIDD when subjected to a His-tag pull-down assay followed by SDS-PAGE (Fig. 2C, 2D). The gel-filtration profile confirmed that caspase-2 CARD was not solubilized by and did not interact with co-expressed full-length RAIDD. Taken together, the results of this study and previous studies demonstrate that intact RAIDD can interact with PIDD DD, but not caspase-2 CARD, despite interaction of the separated domains of RAIDD, RAIDD CARD, and RAIDD DD with caspase-2 CARD and PIDD DD.

Caspase-2 CARD can be solubilized and interact with full-length RAIDD in the presence of PIDD DD

Full-length adaptor proteins that contain two death domain superfamilly members such as RAIDD and FADD have often been reported to fail to interact with their binding partner in intact form (41). These adapter proteins have evolved many regulatory elements to avoid accidental induction of cell death, including conformational changes. Because full-length RAIDD did not interact with caspase-2 CARD, but did interact with PIDD DD, we investigated whether full-length RAIDD could interact with caspase-2 in the presence of PIDD DD. To accomplish this, we performed a His-tag pull-down assay using samples containing co-expressed RAIDD with His-tag, PIDD DD with His-tag and caspase-2 CARD without His-tag. During analysis, all 3 proteins were detected upon SDS-PAGE, indicating that caspase-2 CARD can be solubilized and interact with full-length RAIDD in the presence of PIDD DD (Fig. 3A). When the buffer condition of 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl was used for gel filtration chromatography, a complex peak centered at approximately 9.5 mL in a Superdex 200 column was obtained (Fig. 3B). Because the individual components would have elution peaks at around 16-18 mL and the complex containing RAIDD and PIDD DD would have elution peaks at around 12 mL, the much larger molecular complex containing all three proteins should correspond to the ternary complex of PIDD DD: RAIDD: caspase-2 CARD.
The SDS-PAGE loaded with the peak fractions obtained from the ternary complex confirmed that it contains 3 proteins, RAIDD, PIDD DD, and caspase-2 CARD (Fig. 3B).

**Model of the mechanism of PIDDosome assembly**

Finally, we proposed a model of the process of PIDDosome assembly. RAIDD may exist in equilibrium between the open and closed states. Under normal conditions, the closed form of RAIDD might be dominant. It has been suggested that the existence of closed forms of adaptor molecules for apoptosis, such as FADD and RAIDD is responsible for fine control of important cellular process (38, 42). Upon genotoxic stress, RAIDD favors the open conformation and binds to PIDD first via DD:DD interaction to allow PIDDosome formation. Interaction of PIDD to RAIDD stabilizes the open conformation of RAIDD, after which caspase-2 can be recruited to the complex via a CARD:CARD interaction (Fig. 4). The results of the present study will be useful for elucidation of the order of PIDDosome assembly.

**MATERIALS AND METHODS**

**Protein expression and purification**

Previously reported clones for the expression of RAIDD (amino acid residue 1-199), RAIDD DD (amino acid residue 94-199), RAIDD CARD (amino acid residue 1-92), and PIDD DD (amino acid residue 777-883) were used (38). The cDNA of mouse caspase-2 was used as a template for PCR and cloned into pOKD plasmid vector that was made in house to add or remove the His-tag. Caspase-2 CARD contains amino acids residues 18 to 164.

Recombinant full-length RAIDD, RAIDD CARD, RAIDD DD, and PIDD DD were expressed in *E. coli* BL21 (DE3) RILP and purified as previously described (38). Caspase-2 CARD was expressed in the BL21 (DE3) *E. coli* line. The purification process for all target proteins was similar to the process used for RAIDD DD and PIDD DD purification (43). Briefly, expression was induced by treatment with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 20°C. Then, the bacteria were collected, resuspended and lysed by sonication in 80 mL lysis buffer (20 mM Tris-HCl at pH 7.9, 500 mM NaCl, 10 mM imidazole, and 5 mM β-ME). Next, the cell debris was removed by centrifugation at 16,000 rpm for 1 h at 4°C. The His-tagged target was purified subsequently by affinity chromatography using Ni-NTA beads (Qiagen) and then subjected to gel-filtration chromatography using S-200 (GE healthcare) pre-equilibrated with buffer containing 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl.

**Pull-down assay**

A coexpression system was used to conduct a pull-down assay. RAIDD and RAIDD CARD in pET 26b vector were co-transformed with caspase-2 CARD in pOKD vector separately into BL21(DE3) *E. coli* competent cells. Expression was then induced with 0.5 mM IPTG overnight at 20°C. The cells were subsequently collected and lysed by sonication in lysis buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl, 10 mM imidazole), after which the lysate was removed by centrifugation and the supernatant fractions were applied to a gravity-flow column (Bio-Rad) packed with Ni-NTA affinity beads (Qiagen). The unbound bacterial proteins were removed from the column using washing buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl, 60 mM imidazole, 10% glycerol). For the ternary complex test, PIDD DD expressed cells were mixed with RAIDD:caspase-2 CARD co-expressed cells and lysed by sonication in lysis buffer. Complex formation was detected by SDS-PAGE.

**Complex assay with gel-filtration chromatography**

For gel filtration analysis to detect complex formation, protein samples from a pull-down assay were applied to a gel-filtration column (Superdex 200 HR 10/30, GE healthcare) that had been pre-equilibrated with a solution of 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. Then, the fractions were collected and subjected to SDS-PAGE. The same method was used to detect ternary complex formation.

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