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The structure of the CARD8 caspase-recruitment domain suggests its association with the FIIND domain and procaspases through adjacent surfaces

CARD8 plays crucial roles in regulating apoptotic and inflammatory signaling pathways through the association of its caspase-recruitment domain (CARD) with those of procaspase-9 and procaspase-1. The CARD8 CARD has also been predicted to form an intramolecular complex with its FIIND domain. Here, the first crystal structure of the CARD8 CARD is reported; it adopts a six-helix bundle fold with a unique conformation of the $\alpha 6$ helix that is described here for the first time. The surface of the CARD8 CARD displays a prominent acidic patch at its $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices that may interact with the procaspase-9 CARD, whereas an adjacent charged surface at its $\alpha 3$ and $\alpha 4$ helices may associate with the CARD8 FIIND domain without interfering with the CARD–CARD interaction. This suggests that the function of CARD8 may be regulated by both intramolecular and intermolecular interactions involving electrostatic attractions.

1. Introduction

The caspase-recruitment domain (CARD) is a member of the death-domain superfamily that assembles key signaling pathways during cell death and inflammation through homotypic interactions (Hofmann *et al.*, 1997; Kersse *et al.*, 2011). Many CARD-containing proteins also exert regulatory functions that modulate these signaling pathways. One such important regulator is the cytosolic protein CARD8/TUCAN/Cardinal/NDPP1 (Bouchier-Hayes *et al.*, 2001; Pathan *et al.*, 2001; Razmara *et al.*, 2002; Zhang & Fu, 2002). CARD8 has a C-terminal CARD preceded by a FIIND (function to find domain), which is a unique domain structure that is shared with the inflammasome-forming NOD-like receptor molecule NLRP1 (Tschopp *et al.*, 2003) and which may have evolved from a common ancestor (Bagnall *et al.*, 2008). Human genetic studies have suggested that variants of CARD8 are associated with chronic inflammatory disorders (McGovern *et al.*, 2006; Franke *et al.*, 2007; Büning *et al.*, 2008; Schoultz *et al.*, 2009; Roberts *et al.*, 2010), and high levels of CARD8 expression have been observed in several tumor cell lines and malignant specimens from human patients (Bouchier-Hayes *et al.*, 2001; Pathan *et al.*, 2001; Razmara *et al.*, 2002; Zhang & Fu, 2002), underlying its importance in regulating host immune responses and maintaining homeostasis.

Both the CARD and the FIIND domains of CARD8 have been reported to mediate its regulatory function. The FIIND domain of CARD8 inhibits NF- κ B activation, possibly through interaction with IKK γ (Bouchier-Hayes *et al.*, 2001). It may also bind the nucleotide-binding domain (NBD) domain of NOD2 and NLRP3 to regulate the immune response to bacterial infections (Kampen *et al.*, 2010) and inflammasome assembly (Agostini *et al.*, 2004). Interestingly, the FIIND domains of both CARD8 and NLRP1 have been reported to undergo autocleavage as a mechanism to regulate inflammasome formation and innate immune responses (D’Osualdo *et al.*, 2011). The CARD8 CARD has been shown to associate with procaspase-9 (Pathan *et al.*, 2001) and procaspase-1 (Razmara *et al.*, 2002) through

CARD–CARD interactions to suppress apoptosis and inflammatory responses, respectively (Pathan *et al.*, 2001; Razmara *et al.*, 2002; Stilo *et al.*, 2002; Agostini *et al.*, 2004); however, the mechanisms of such associations remain unclear. The CARD8 and NLRP1 CARDS have also been predicted to form intramolecular complexes with their FIIND domains through charge–charge interactions (D’Osualdo *et al.*, 2011), which may represent a unique mechanism to modulate the functions of these two FIIND–CARD-containing proteins.

Here, we report the first crystal structure of the CARD8 CARD as a fusion protein with maltose-binding protein (MBP). The structure reveals a distinct conformation of the CARD8 CARD $\alpha 6$ helix that has not been described previously. Examination of the electrostatic charge surface of the CARD8 CARD suggests that electrostatic attraction may play an important role in its association with the procaspase-9 CARD as well as with its own FIIND domain.

2. Methods

2.1. Protein expression and purification

The human CARD8 CARD (NCBI accession No. NP_001171829; residues 451–537) was cloned into a pET30a-derived vector with a

noncleavable N-terminal MBP tag. The MBP tag harbors mutations (D82A/K83A/E172A/N173A/K239A) designed to enhance its crystallization propensity (Moon *et al.*, 2010). Transformed *Escherichia coli* BL21 (DE3) CodonPlus RIPL cells (Stratagene, Santa Clara, California, USA) were grown at 310 K and induced at 291 K with 0.2 mM IPTG for 4 h. The cells were lysed by sonication in buffer A (20 mM Tris–HCl pH 8.0, 100 mM NaCl) plus 5 mM imidazole, DNase (Biomatik, Wilmington, Delaware, USA) and protease inhibitors (Roche Applied Science, Indianapolis, Indiana, USA). The soluble protein was purified from the cleared lysate using a HisPrep IMAC column (GE Healthcare Biosciences, Piscataway, New Jersey, USA). The IMAC-eluted MBP–CARD protein was further purified using size-exclusion chromatography.

2.2. Crystallization

The purified MBP–CARD protein was concentrated to 50 mg ml^{−1} using Amicon centrifugal concentrators (Millipore, Billerica, Massachusetts, USA) before setting up hanging drops using a Mosquito crystallization robot (TTP LabTech, UK). Crystals grew within 24 h using a solution consisting of 16% PEG 8000, 0.1 M NaI, 0.1 M sodium acetate pH 5.2, 20%(v/v) glycerol, 5%(v/v) ethylene

Figure 1

(a) Sequence alignment of the CARDS from CARD8, NLRP1, Apaf-1 and procaspase-9 (Casp9). The six α -helices of the CARD structures are underlined and are marked above the sequences. The residues that are conserved among the CARDS are shaded yellow and those that are conserved in the CARD8 and NLRP1 CARDS are shaded green. The acidic residues of Apaf-1 CARD that are important for its association with the procaspase-9 CARD are colored red. The acidic residues of CARD8 CARD that form a prominent negatively charged surface patch near the $\alpha 2$ – $\alpha 3$ helices are colored red. The basic residues of procaspase-9 that are essential for Apaf-1 binding are colored blue. (b) The MBP–CARD structure is shown as ribbons with MBP colored gray and the CARD colored orange. The six α -helices of the CARD are labeled. The bound maltose at the MBP is shown in stick representation. (c) Details of the MBP–CARD interface are shown with hydrogen bonds displayed as gray dotted lines.

Table 1

X-ray diffraction data-collection and structure-refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$P6_522$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 94.9$, $c = 219.4$, $\alpha = \beta = 90$, $\gamma = 120$
Wavelength ($\text{\AA}$)	1.033
Resolution ($\text{\AA}$)	50–2.46 (2.49–2.46)
No. of reflections (total/unique)	422662/21951
Multiplicity	19.3 (18.2)
Completeness (%)	99.8 (99.6)
$\langle I/\sigma(I) \rangle$	33.8 (3.6)
$R_{\text{merge}}^\dagger$ (%)	7.2 (94.5)
$R_{\text{p.i.m.}}^\ddagger$ (%)	1.7 (22.2)
Refinement	
Resolution ($\text{\AA}$)	50–2.46
No. of protein atoms	3601
No. of heteroatoms	102
B factors ($\text{\AA}^2$)	
Protein atoms	79.1
Heteroatoms	64.2
R.m.s.d. bond lengths ($\text{\AA}$)	0.004
R.m.s.d. bond angles ($^\circ$)	0.853
$R_{\text{work}}^\S$ (%)	20.1
$R_{\text{free}}^\P$ (%)	25.3
Ramachandran plot favored/disallowed †† (%)	96.7/0.0
PDB code	4ikm

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the i th and the mean measurement of the intensity of reflection hkl . $^\ddagger R_{\text{p.i.m.}} = \sum_{hkl} [1/[N(hkl) - 1]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|] / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the i th and the mean measurement of the intensity of reflection hkl and $N(hkl)$ is the redundancy of reflection hkl . $^\S R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. No I/σ cutoff was applied. $^\P R_{\text{free}}$ is the R value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set that was excluded from refinement. †† Values from *MolProbity* (<http://molprobity.biochem.duke.edu/>).

glycol and 1% (w/v) maltose were added to the crystallization solution before flash-cooling the crystals in liquid nitrogen for X-ray diffraction data collection.

2.3. X-ray diffraction, structure determination and refinement

X-ray diffraction data were collected on the GM/CA-CAT beamlines at the Advanced Photon Source, Argonne National Laboratory. Data were processed with the *HKL-2000* program suite (Otwinowski & Minor, 1997). The structure was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* program suite (Winn *et al.*, 2011). A structure of MBP from the Protein Data Bank (PDB entry 3vd8; T. Jin, A. Perry, P. Smith & T. S. Xiao, unpublished work) was used as the search model. Electron-density maps calculated with phases from the MBP search model

clearly showed excellent density for the CARD8 CARD. Model building was carried out with *Coot* (Emsley & Cowtan, 2004) and refinement was carried out with *phenix.refine* (Adams *et al.*, 2010). The final structure contained 463 residues, of which residues Ala372–Leu458 correspond to residues Ala451–Leu537 of CARD8 (NP_001171829). Validation using the *MolProbity* server (Chen *et al.*, 2010) gave a *MolProbity* score of 1.49, which is in the 99th percentile of structures with comparable resolutions. 97.2% of the protein residues are in favored regions of the Ramachandran plot, with no outliers. Electrostatic surfaces were calculated using the program *DelPhi* (v.4; Honig & Nicholls, 1995) and displayed with *PyMOL* (<http://www.pymol.org>).

3. Results and discussion

3.1. Determination of the CARD8 CARD structure

Sequence alignment of several CARDS reveals that most sequence conservations are of hydrophobic residues that are likely to be buried in the hydrophobic core of the domains (Fig. 1a). In addition, the CARD8 CARD has higher sequence similarity to the NLRP1 CARD. In order to reveal the molecular architecture of the CARD8 CARD, we pursued crystallographic studies. Overexpressed recombinant CARD8 CARD underwent severe aggregation that hindered purification and crystallization. To overcome this, we adopted a fusion-protein strategy to covalently link an MBP to the CARD8 CARD to maintain solubility and facilitate crystallization. This is similar to the method previously employed to determine the structure of the IPS-1 CARD (Potter *et al.*, 2008). The fusion protein was successfully crystallized and the statistics for X-ray diffraction data collection and structural refinement are given in Table 1. The $\alpha 2$ – $\alpha 3$ loop and the $\alpha 5$ and $\alpha 6$ helices of the CARD8 CARD are located at its hydrophilic interface with the MBP, with six hydrogen bonds contributed by four CARD8 residues (Figs. 1b and 1c). This may stabilize the relative positions of the MBP and CARD, thus facilitating crystallization.

3.2. The CARD8 and NLRP1 CARDS contain conformationally distinct $\alpha 6$ helices

The CARD8 CARD contains six antiparallel α -helices folded into a compact globular domain (Fig. 2a), similar to other members of the death-domain superfamily (Park *et al.*, 2007; Kersse *et al.*, 2011). A structural homology search using the *PDBFold* server (Krissinel & Henrick, 2004) revealed similarities between the CARD8 CARD and those of NLRP1, NOD1, Apaf-1, ICEBERG and procaspase-9 (Table 2). Superposition of the structures demonstrates that the

Figure 2

(a) The CARD8 CARD structure is shown as orange ribbons. (b) The CARD structures from CARD8 (orange), NLRP1 (pink), Apaf-1 (cyan) and procaspase-9 (green) are superimposed and displayed as ribbons. The view is the same as in (a). The view in (c) is rotated approximately 90° along the horizontal axis from that in (b).

Table 2

Comparison of the CARD8 CARD structure (PDB entry 4ikm) with other CARD structures using the *PDBFold* server (<http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmsserver>).

CARD (PDB code)	Z score	R.m.s.d. (Å)	Aligned residues	Identity (%)
NLRP1 (3kat)	8.9	1.2	78	32
NOD1 (2nz7)	8.6	1.2	75	19
Apaf-1 (1cy5)	8.3	1.4	77	18
ICEBERG (1dgn)	7.5	1.5	79	24
Procaspase-9 (3ygs)	7.0	2.0	75	15
RIG-I (2lwe)	5.9	2.0	71	17

$\alpha 1$ – $\alpha 5$ helices of the CARDs can be superimposed well, with slight variations in the length and orientation of each helix (Figs. 2*b* and 2*c*). In contrast, the $\alpha 6$ helices of the CARD8 and NLRP1 CARDs appear to be located away from the bulk of the CARD structure, with their helical axes about 45° from those of the Apaf-1 and procaspase-9 CARDs (Figs. 2*b* and 3*a*). It is unlikely that the MBP fusion tag played a significant role in causing this structural difference, because only one residue, Arg533, of the CARD8 CARD $\alpha 6$ helix is in contact with the MBP fusion tag (Fig. 1*c*) and its conformation is very similar to that of the NLRP1 CARD (Figs. 2*b* and 3*a*). A close examination of the interactions between the $\alpha 6$ helices and their structural neighbors reveals that the $\alpha 6$ -helix residues Val529 in CARD8 and Ile1456 in NLRP1 interact with the conserved $\alpha 1$ -helix residues Phe453 and Phe1381, respectively (Figs. 3*b*, 3*c* and 1*a*). These hydrophobic contacts in effect buttress the $\alpha 6$ helices away from the main body of the CARD structure. In contrast, the Apaf-1 and procaspase-9 CARDs contain smaller residues in the equivalent $\alpha 1$ and $\alpha 6$ helices, thus explaining the ability of their $\alpha 6$ helices to pack more closely to the bulk of the domain (Figs. 3*d* and 3*e*). The functional significance of these different $\alpha 6$ -helix conformations remains to be explored.

3.3. The CARD8 CARD exhibits charged surface patches that may mediate its interaction with partner domains

The death-domain superfamily members are known to display distinctive charged surface patches that contribute to their homotypic associations (Park *et al.*, 2007). Our analysis of the CARD8 CARD structure demonstrates that it also displays a prominent acidic surface patch near its $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices (Fig. 4*a*). In the structure of the Apaf-1 CARD–procaspase-9 CARD complex, the acidic $\alpha 2$ – $\alpha 3$ helices of the Apaf-1 CARD bind to the basic $\alpha 1$ and $\alpha 4$ helices of the procaspase-9 CARD (Qin *et al.*, 1999). Similarly, the RAIDD CARD and the procaspase-2 CARD were predicted to associate through complementary electrostatic surfaces (Chou *et al.*, 1998). Even though the Apaf-1 acidic interface residues are not strictly conserved in the CARD8 CARD, the latter possesses a number of acidic residues in the equivalent $\alpha 2$ – $\alpha 3$ helices (Fig. 1*a*). As a result, the prominent acidic surface of the CARD8 CARD may interact with the highly positively charged $\alpha 1$ and $\alpha 4$ helices of the procaspase-9 CARD in a similar mode to that observed in the structure of the Apaf-1–procaspase-9 complex. Future structural studies of the CARD8–procaspase-9 complex will be required to reveal the details of this CARD–CARD interaction.

Through modeling studies, D'Ossualdo and coworkers reported that the CARD8 CARD may form an intramolecular complex with its FIIND through charge–charge interactions (D'Ossualdo *et al.*, 2011). In this model, the $\alpha 3$ and $\alpha 4$ helices of the CARD8 CARD associate with the FIIND through several salt bridges. In agreement with this, the electrostatic surface of the CARD8 CARD shows mixed negative and positive charges at the $\alpha 3$ and $\alpha 4$ helices (Fig. 4*b*).

Superposition of the CARD8 model and the Apaf-1–procaspase-9 structure onto the CARD8 CARD crystal structure reveals that the CARD8 CARD may interact with procaspase-9 and with its FIIND through adjacent surfaces near the $\alpha 3$ helix without causing steric hindrance (Figs. 4*c* and 4*d*). This suggests that the function of CARD8 may be modulated by both intramolecular and intermolecular interactions.

4. Conclusions

The crystal structure of the CARD8 CARD at 2.46 Å resolution illustrates a six-helix bundle fold with a distinct conformation of the $\alpha 6$ helix that has not been described previously. The structure reveals a prominent acidic surface patch at the $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices which may be employed as an interface with the procaspase-9 CARD to form a CARD–CARD complex similar to the Apaf-1–procaspase-9 structure. The mixed negative and positive charges at the $\alpha 3$ and $\alpha 4$ helices may be involved in the intramolecular interaction with the FIIND without interfering with the CARD–CARD association. Overall, the structure of the CARD8 CARD suggests that it may

Figure 3
(*a*) Superposition of the $\alpha 5$ – $\alpha 6$ helices of the CARD8 (orange), NLRP1 (pink), Apaf-1 (cyan) and procaspase-9 (green) CARDs. The orientation of the $\alpha 6$ helices differ by $\sim 45^\circ$ between the CARD8/NLRP1 and Apaf-1/procaspase-9 CARDs. (*b*) The $\alpha 5$ – $\alpha 6$ helices of the CARD8 CARD are shown as ribbons and a portion of the $\alpha 1$ helix is shown as a C^α trace, with the Val529 and Phe453 side chains displayed as spheres. Similar views and equivalent residues are shown for the NLRP1 (pink) (*c*), Apaf-1 (cyan) (*d*) and procaspase-9 (green) (*e*) CARDs.

Figure 4

(a) The electrostatic charge surface and C^α trace of the CARD8 CARD is shown with the $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices facing the viewer. The scale is $-5kT/e$ (red) to $5kT/e$ (blue). The locations of the helices that contribute to the surface charges are indicated by arrows. (b) The charge surface and C^α trace of the CARD8 CARD is shown with the $\alpha 3$ – $\alpha 4$ helices facing the viewer. (c) The crystal structure (PDB entry 3ygs; Qin *et al.*, 1999) of the Apaf-1 CARD (cyan) in complex with the procaspase-9 CARD (green) is superimposed onto the CARD8 CARD crystal structure (orange) using the Apaf-1 and CARD8 CARD structures. The CARD8 FIIND–CARD model is also superimposed onto the CARD8 CARD crystal structure, with its FIIND shown as a gray surface. The view on the right is rotated approximately 90° along the horizontal axis from that on the left. Helices at the CARD–CARD and CARD–FIIND interfaces are labeled.

associate with its FIIND and with procaspases through adjacent surfaces to regulate apoptotic and inflammatory signaling pathways.

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