Tengchuan Jin, Mo Huang,
Patrick Smith, Jiansheng Jiang
and T. Sam Xiao*

Structural Immunobiology Unit, Laboratory of
Immunology, National Institute of Allergy and
Infectious Diseases, National Institutes of
Health, 4 Memorial Drive, Building 4,
Room 228, Bethesda, MD 20892-0430, USA

Correspondence e-mail: xiaot@niaid.nih.gov

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Structure of the caspase-recruitment domain from a zebrafish guanylate-binding protein

The caspase-recruitment domain (CARD) mediates homotypic protein–protein interactions that assemble large oligomeric signaling complexes such as the inflammasomes during innate immune responses. Structural studies of the mammalian CARDS demonstrate that their six-helix bundle folds belong to the death-domain superfamily, whereas such studies have not been reported for other organisms. Here, the zebrafish interferon-induced guanylate-binding protein 1 (zIGBP1) was identified that contains an N-terminal GTPase domain and a helical domain typical of the mammalian guanylate-binding proteins, followed by a FIIND domain and a C-terminal CARD similar to the mammalian inflammasome proteins NLRP1 and CARD8. The structure of the zIGBP1 CARD as a fusion with maltose-binding protein was determined at 1.47 Å resolution. This revealed a six-helix bundle fold similar to the NLRP1 CARD structure with the bent $\alpha 1$ helix typical of all known CARD structures. The zIGBP1 CARD surface contains a positively charged patch near its $\alpha 1$ and $\alpha 4$ helices and a negatively charged patch near its $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices, which may mediate its interaction with partner domains. Further studies using binding assays and other analyses will be required in order to address the physiological function(s) of this zebrafish protein.

1. Introduction

The caspase-recruitment domain (CARD) was first described as an essential signaling module in apoptosis (Hofmann *et al.*, 1997). It is now recognized as a domain that mediates protein–protein interactions in the assembly of inflammasomes (Martinon *et al.*, 2002) and apoptosomes (Qi *et al.*, 2010), which are large oligomeric signaling platforms implicated in development, cancer and innate immune responses. Several mammalian receptor molecules from the NOD-like receptor (NLR) family such as NLRP1 and NLRP3 are known to form inflammasomes. In addition to the receptor molecules, inflammasomes also contain the apoptosis-associated speck-like protein containing a CARD (ASC) as an adapter molecule and the effector enzyme procaspase-1 (Martinon *et al.*, 2002). Structurally, the inflammasome receptors associate with the adapter ASC through their respective pyrin domains (PYDs), which in turn recruit procaspase-1 through their CARDS. In addition to PYD, human NLRP1 also contains a CARD that directly binds the procaspase-1 CARD (Martinon *et al.*, 2002). Both CARDS and PYDs belong to the death-domain (DD) superfamily of signaling domains, which also includes the death-effector domains (DEDs), and all adopt six-helix bundle folds with subfamily-specific structural features (Park, Lo *et al.*, 2007; Kersse *et al.*, 2011). For example, the PYDs have short $\alpha 3$ helices and long $\alpha 2$ – $\alpha 3$ loops, whereas the CARDS contain bent $\alpha 1$ helices (Kersse *et al.*, 2011).

The death-domain superfamily members form signaling complexes through three types of homotypic interface (Weber & Vincenz, 2001; Ferrao & Wu, 2012). For example, the DD has been reported to form oligomeric complexes such as the Myddosome and PIDDosome through type I, II and III interactions (Park, Logette *et al.*, 2007; Lin *et al.*, 2010). In contrast, DEDs and CARDS have only been reported to form dimeric complexes through the type I interaction, which is characterized by electrostatic interactions between the $\alpha 1$ and $\alpha 4$

helices of one domain and the $\alpha 2$ – $\alpha 3$ helices of the other (Qin *et al.*, 1999; Yang *et al.*, 2005).

Because of their essential functions, many CARD-containing proteins are conserved through evolution. In particular, orthologs of several NLRs and ASC have been identified in non-mammals such as the model organism zebrafish (*Danio rerio*) and were proposed to play important roles in the zebrafish innate immune system (Stein *et al.*, 2007; Laing *et al.*, 2008). In an effort to identify zebrafish proteins with sequence homology to human inflammasome receptors containing CARD, we searched the zebrafish proteome with the human NLRP1 CARD sequence and identified several putative CARD-containing proteins. Of particular interest is the interferon-induced guanylate-binding protein 1 (zIGBP1; gi:326665123), the C-terminal CARD of which is preceded by a FIIND domain, a unique domain structure that is shared with the mammalian NLRP1 and CARD8 proteins involved in inflammasome formation (Martinon *et al.*, 2002; Tschopp *et al.*, 2003) but that has not been characterized in zebrafish proteins. Since there has been no report of any CARD structure from zebrafish proteins, we initiated crystallographic studies of the zIGBP1 CARD as a first step in understanding the structure and function of this CARD-containing zebrafish protein.

2. Methods

2.1. Identification of zIGBP1

zIGBP1 was identified using the sequence of the human NLRP1 CARD (gi:14719829) and the protein BLAST (BLASTP) server

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>; Altschul *et al.*, 2005) against the zebrafish protein database. Among the top hits, zIGBP1 (gi:326665123) has an *E* value of 1×10^{-5} (35% identity and 48% similarity), zCARD-rel (gi:194353931) has an *E* value of 5×10^{-7} (36% identity and 52% similarity) and zASC (gi:70912376) has an *E* value of 1×10^{-5} (31% identity and 61% similarity) (see Fig. 1). A reciprocal BLASTP search using the zIGBP1 CARD against the human protein database identified human NLRP1 (*E* value 2×10^{-5} ; 35% identity and 48% similarity) and human ASC (gi:10835256; *E* value 2×10^{-7} ; 35% identity and 52% similarity).

2.2. Molecular cloning, protein expression and purification

The coding sequence for residues Ser872–Gln964 of the zIGBP1 CARD was amplified from a zebrafish cDNA library with Phusion High-Fidelity DNA polymerase (NEB, Ipswich, Massachusetts, USA) using a forward primer (5'-CTAGCGGCCGCTAGTGAATTGTG-GATGCCCTCAGAGGTGATC-3') containing an *NotI* restriction site (bold) and a reverse primer (5'-CTACTCGAGCTGAGGACC-GCTGGATTCTGTGTCTAG-3') containing an *XhoI* restriction site (bold). The cDNA was cloned into a pET30a vector with a noncleavable N-terminal MBP tag designed to facilitate crystallization (Ullah *et al.*, 2008; Moon *et al.*, 2010). Transformed *Escherichia coli* BL21 (DE3) CodonPlus RIPL cells (Stratagene, Santa Clara, California, USA) were grown at 310 K and induced with 0.2 mM IPTG at 291 K for 4 h, harvested and lysed in buffer A (20 mM Tris–HCl pH 8.0, 100 mM NaCl) plus 5 mM imidazole, DNase (Biomatix, Wilmington, Delaware, USA) and protease inhi-

Figure 1 zIGBP1 contains GD, HD, FIIND and CARD domains. (a) The domain arrangements of zIGBP1 and zCARD-rel are shown with the residue range for each domain marked below. (b) Sequence alignment of the FIIND domains near its autocleavage site, which is marked by an inverted triangle. The histidine and serine residues implicated in the cleavage are shaded yellow and other conserved residues are shaded gray. (c) Sequence alignment of zIGBP1, zCARD-rel, zASC and hNLRP1. Conserved residues are shaded gray. The six α -helices are marked above the sequences.

bitors (Roche Applied Science, Indianapolis, Indiana, USA). Soluble protein from the cell lysate was purified using a HisPrep IMAC column (GE Healthcare Life Sciences, Piscataway, New Jersey, USA) and further purified using an XK26/60 Superdex 200 size-exclusion column in buffer *A* supplemented with 5 mM maltose (Research Products International Corp., Mount Prospect, Illinois, USA).

2.3. Crystallization

Purified MBP-CARD protein was concentrated to 40 mg ml⁻¹ before setting up hanging drops for vapor-diffusion crystallization. Multiple commercial crystallization screens were tested using a Mosquito crystallization robot (TTP LabTech, United Kingdom). The best-diffracting crystals were grown with a well solution consisting of 20% PEG 1000, 0.2 M sodium malonate, 0.1 M MES-Na pH 6.0 in one week. 20% (v/v) ethylene glycol was added to the reservoir solution as a cryoprotectant before flash-cooling the crystals in liquid nitrogen for X-ray diffraction data collection.

2.4. X-ray diffraction, structure determination and refinement

X-ray diffraction data were collected at the GM/CA-CAT of the Advanced Photon Source, Argonne National Laboratory. Data were processed with *HKL-2000* (Otwinowski & Minor, 1997) and *XD5* (Kabsch, 2010). The crystal belonged to space group *P*₂₁₂₁₂₁, with unit-cell parameters *a* = 65.8, *b* = 124.8, *c* = 178.0 Å. The solvent content was estimated to be 47%, with a Matthews coefficient of 2.33 Å³ Da⁻¹, for three molecules of the fusion protein in the asymmetric unit. Analysis of the X-ray diffraction data using *phenix.xtriage* from the *PHENIX* package (Adams *et al.*, 2010) revealed a

significant off-origin peak in the Patterson map at (0, 0.333, 0) with a height corresponding to 70% of the origin peak. This indicates strong pseudo-translational noncrystallographic symmetry (tNCS). Molecular replacement using *Phaser* (McCoy *et al.*, 2007) with an MBP structure (PDB entry 3vd8; Jin *et al.*, 2012) as a search model identified three copies of MBP in the asymmetric unit, with a top LLG (log-likelihood gain) of 3378. Electron-density maps calculated with the three MBP molecules clearly showed the presence of three CARDs, which were manually built with *Coot* (Emsley *et al.*, 2010) and refined with *PHENIX* (Adams *et al.*, 2010).

The three MBP-CARD fusion molecules (*A*, *B* and *C*) in the asymmetric unit are related to each other largely by translation along the *b* axis (see Fig. 2*b*), together with a small rotational component in the NCS operators. Specifically, the second molecule (*B*) is related to the first molecule (*A*) by the following NCS operator: rotation (1.0000, 0.0028, 0.0010, -0.0028, 0.9994, -0.0333, -0.0011, 0.0333, 0.9994) and fractional translation (-0.0202, 0.3309, 0.0030), with an r.m.s.d. of 0.32 Å for 461 matched C^α atoms. The rotation matrix is equivalent to polar rotation angles (*φ*, *ψ* and *κ*) of 358.2°, 90° and 358.0°, respectively. The third molecule (*C*) is related to the first molecule (*A*) by the following NCS operator: rotation (1.0000, 0.0014, 0.0032, -0.0012, 0.9988, -0.0487, -0.0033, 0.0487, 0.9988) and fractional translation (-0.0202, 0.6671, 0.0117), with an r.m.s.d. of 0.23 Å for 461 matched C^α atoms. The rotation matrix is equivalent to polar rotation angles (*φ*, *ψ* and *κ*) of 356.2°, 90° and 357.2°, respectively. The relative positions of the MBP and CARD domains of the three fusion molecules in the asymmetric unit are essentially identical. Upon superposition of the MBP structures, the CARD structures are related to each other by hinge rotation angles of 2.6° between

Figure 2

Structure of the MBP-CARD fusion protein. (*a*) A σ_A -weighted $2F_o - F_c$ map contoured at 0.76 e^-3 (1.2σ) near the zIGBP1 CARD $\alpha 3$ helix is shown as a blue mesh. (*b*) The packing of the three MBP-CARD fusion molecules (magenta, green and blue) in the crystallographic asymmetric unit and their symmetry mates (gray) are shown along with the three axes of the unit cell. (*c*) The MBP-CARD structure is shown as ribbons with the MBP colored gray and the CARD colored magenta. (*d*) Details of the MBP-CARD interface are shown with hydrogen bonds displayed as gray dotted lines.

molecule *A* and molecule *B* and of 2.2° between molecule *A* and molecule *C* as calculated by *HINGE* (http://exon.niaid.nih.gov/sis/hinge_file.html). The average temperature factors for the three molecules in the asymmetric unit are 18.0, 19.2 and 21.6 Å², respectively. Analysis of the quaternary structures using the *PISA* server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) demonstrated that within the crystallographic asymmetric unit, packing of the three molecules buries ~700 Å² of solvent-accessible surface area for each molecule. This is largely mediated by the MBP structure. In comparison, the largest interface between the crystallographically unique molecules and their symmetric mates buries ~1560 Å² of solvent-accessible surface area. This is mediated by both the MBP and the CARD structures.

In addition to the three MBP-CARD structures and 1922 water molecules, there are three maltotriose molecules at the MBP sugar-binding sites, three malonate ions from the crystallization solution and five ethylene glycol molecules from the cryoprotectant. Residues Ser376–Ser462 of the fusion protein correspond to residues Ser872–Ser958 of the zIGBP1 CARD. Validation by the *MolProbity* server (Chen *et al.*, 2010) showed a *MolProbity* score of 1.09, which is in the 99th percentile of structures of comparable resolution, with 98.7% of all protein residues in the favored regions of the Ramachandran plot and no outliers. Electrostatic surfaces were calculated with *DelPhi* (v.4; Honig & Nicholls, 1995) and were displayed with *PyMOL* (Schrödinger, New York, USA).

3. Results and discussion

3.1. Identification of zIGBP1

While we were studying the human NLRP1 inflammasome receptor, we searched for CARD-containing zebrafish proteins using *BLASTP* and found several candidates. These included zASC, zIGBP1 and zCARD-rel, which appears to be a truncated variant of zIGBP1. Both zIGBP1 and zCARD-rel contain a C-terminal CARD preceded by a FIIND domain (Figs. 1a–1c), which is a unique domain configuration not previously characterized for zebrafish proteins. In mammals, both NLRP1 and CARD8 proteins share this domain arrangement and their FIIND domains have been reported to undergo autoproteolytic cleavage at a serine residue facilitated by an N-terminal histidine residue (D'Osualdo *et al.*, 2011). This was proposed as a potential regulatory mechanism for inflammasome activation and innate immune responses. Interestingly, the histidine and serine residues implicated in mammalian FIIND-domain autocleavage are conserved in zIGBP1 and zCARD-rel (Fig. 1b), suggesting that a similar proteolytic event could also regulate these zebrafish proteins.

In addition to the FIIND and CARD domains, zIGBP1 also contains an N-terminal GTPase domain (GD) and a helical domain (HD) (Fig. 1a) typical of the guanylate-binding proteins (GBPs), a family of interferon-induced immune-response proteins that initiate effector mechanisms to control infections (Cheng *et al.*, 1983; MacMicking, 2004; Kim *et al.*, 2012). Recently, Shenoy and coworkers reported that the GTPase domain of mammalian GBP5 binds the PYD of the inflammasome receptor NLRP3, which promotes NLRP3 inflammasome assembly in response to pathogenic bacterial infection (Shenoy *et al.*, 2012). Whether the GTPase domain of zIGBP1 interacts with DD superfamily members from the zebrafish NLRs or with its own CARD remains to be determined, as do the roles that zIGBP1 plays in zebrafish antimicrobial immune responses.

Table 1

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

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = 65.8, <i>b</i> = 124.8, <i>c</i> = 178.0
Wavelength (Å)	1.033
Resolution (Å)	50–1.47 (1.50–1.47)
No. of reflections (total/unique)	3208684/237593 (95937/12964)
Completeness (%)	95.7 (74.9)
<i>I</i> / <i>σ</i> (<i>I</i>)	8.50 (1.6)
Average multiplicity	13.50 (7.4)
<i>R</i> _{merge} [†] (%)	22.80 (66.0)
<i>R</i> _{p.i.m.} [‡] (%)	6.1 (26.3)
Refinement	
Resolution (Å)	50–1.47 (1.50–1.47)
No. of protein atoms	10863
Average <i>B</i> factor, protein atoms (Å ²)	19.6
Average <i>B</i> factor, chains <i>A</i> , <i>B</i> , <i>C</i> (Å ²)	18.0, 19.2, 21.6
No. of heteroatoms	2121
Average <i>B</i> factor, heteroatoms (Å ²)	31.9
Wilson <i>B</i> factor (Å ²)	14.2
NCS operators, molecules <i>A</i> – <i>B</i>	
Rotation (φ, ψ, κ) (°)	358.2, 90, 358.0
Translation (fractional)	−0.0202, 0.3309, 0.0030
R.m.s.d. (Å)	0.32
NCS operators, molecules <i>A</i> – <i>C</i>	
Rotation (φ, ψ, κ) (°)	356.2, 90, 357.2
Translation (fractional)	−0.0202, 0.6671, 0.0117
R.m.s.d. (Å)	0.23
R.m.s.d. from ideal bond lengths§ (Å)	0.010
R.m.s.d. from ideal bond angles§ (°)	1.294
<i>R</i> _{work} [¶]	0.188 (0.271)
<i>R</i> _{free} ^{††}	0.224 (0.307)
Validation	
Ramachandran plot favored/disallowed‡‡ (%)	98.7/0.0
Rotamer outliers (%)	0.73
<i>C</i> ^β outliers (%)	0.15
Clashscore	2.95
<i>MolProbity</i> score/percentile	1.09/99th
PDB code	4irl

[†] *R*_{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*. [‡] *R*_{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*. § Values for ideal bond lengths and angles are from Engh & Huber (1991). ¶ *R*_{work} = $\sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$, where *F*_{obs} and *F*_{calc} are the observed and calculated structure factors, respectively. No *I*/*σ*(*I*) cutoff was applied. †† *R*_{free} is the *R* value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement. ‡‡ Values from the *MolProbity* server (<http://molprobity.biochem.duke.edu/>).

3.2. Determination of the zIGBP1 CARD structure

Many death-domain superfamily members are known to form severe aggregates upon overexpression, perhaps partially reflecting their function as protein–protein interaction modules. To investigate the structure of the IGBP1 CARD, we cloned its coding sequence into a bacterial expression vector as a fusion with an MBP-coding sequence. This adopted MBP-fusion strategy was similar to previous efforts to crystallize the human IPS-1 CARD (Potter *et al.*, 2008) employing MBP as a solubility tag, a purification tag and a crystallization chaperone (Koide, 2009). The MBP-fusion protein was successfully expressed and purified to homogeneity and was readily crystallized. The structure was determined by molecular replacement using an MBP structure (PDB entry 3vd8; Jin *et al.*, 2012) as a search model, taking into account a strong pseudo-translational noncrystallographic symmetry (tNCS). The zIGBP1 CARD structure was manually built into the electron-density map calculated with the MBP structures. The data-collection and structure-refinement statistics are given in Table 1 and representative electron density is shown in Fig. 2(a). The packing of the three MBP-CARD fusion protein molecules in the crystallographic asymmetric unit and their symmetry mates are shown in Fig. 2(b). The presence of the MBP fusion is

unlikely to distort the compact domain structure of the zIGBP1 CARD, as the hydrophilic interface between them is composed of seven water-mediated hydrogen bonds involving the zIGBP1 CARD $\alpha 1$ helix and $\alpha 4$ – $\alpha 5$ loop (Figs. 2*c* and 2*d*).

3.3. The surface-charge distribution of the zIGBP1 CARD is similar to those of human CARDs

The structure of the zIGBP1 CARD is composed of six antiparallel α -helices typical of the death-domain superfamily (Park, Lo *et al.*, 2007; Kersse *et al.*, 2011). In particular, it has a bent $\alpha 1$ helix that is characteristic of all known CARD structures from mammalian proteins (Kersse *et al.*, 2011; Figs. 3*a* and 3*b*). A structural homology

Table 2

Comparison of the zIGBP1 CARD structure with other CARD structures using the DALI server.

	Z-score	R.m.s.d. (Å)	Aligned residues	Identity (%)
hNLRP1† (4ifp; Jin, Curry <i>et al.</i> , 2013)	14.7	1.4	85	33
hICEBERG† (1dgn; Humke <i>et al.</i> , 2000)	12.0	2.1	83	23
hNOD1† (2nsn; Coussens <i>et al.</i> , 2007)	12.0	2.0	76	20
hBinCARD† (4fh0; Chen <i>et al.</i> , 2013)	11.5	2.2	85	19
hCIAP1† (3t6p; Dueber <i>et al.</i> , 2011)	11.4	1.7	85	19
hApaf1† (2ygs; Qin <i>et al.</i> , 1999)	11.0	2.7	82	18
mCARMA1‡ (4i16; Li <i>et al.</i> , 2012)	10.4	2.5	82	20
hCaspase9† (3ygs; Qin <i>et al.</i> , 1999)	9.9	3.1	85	18

† Human protein. ‡ Mouse protein.

Figure 3

Overview and surface charge of the zIGBP1 CARD structure. (a) The zIGBP1 CARD structure is colored magenta and superimposed onto the human NLRP1 CARD structure (colored wheat). The six α -helices are labeled. (b) A view rotated by approximately 90° along the horizontal axis from that in (a). (c) The zIGBP1 CARD is displayed as C^α traces and a semi-transparent electrostatic charge surface on a scale of -5 kT/e (red) to 5 kT/e (blue). The view in (d) is rotated along the vertical axis from that in (c). (e, f) The electrostatic charge surface of the human NLRP1 CARD (PDB entry 4ifp; Jin, Curry *et al.*, 2013) is shown similarly as in (c) and (d) for comparison.

search using the DALI server (Holm & Rosenström, 2010) revealed that the zIGBP1 CARD is most similar to the NLRP1 CARD (Jin, Curry *et al.*, 2013), with an r.m.s.d. of 1.4 Å and a sequence identity of 33% (Table 2), suggesting that it likely represents the first CARD structure from a zebrafish protein.

Analysis of the electrostatic charge surface of the zIGBP1 CARD demonstrates that it displays a positively charged surface patch near its $\alpha 1$ and $\alpha 4$ helices and a negatively charged surface near its $\alpha 2$, $\alpha 3$ and $\alpha 5$ helices (Figs. 3c and 3d). This feature is similar to human CARDs such as those from human NLRP1 (Jin, Huang *et al.*, 2013; Figs. 3e and 3f), Apaf-1 (Qin *et al.*, 1999), procaspase-9 (Qin *et al.*, 1999) and RAIDD (Chou *et al.*, 1998). Structural and modeling studies have demonstrated that electrostatic interactions play important roles in mediating Apaf-1 CARD–procaspase-9 CARD and RAIDD CARD–procaspase-2 CARD associations through the type I interaction (Chou *et al.*, 1998; Qin *et al.*, 1999). Furthermore, modeling and structural studies of human CARD8 suggested that electrostatic attraction may mediate the formation of an intramolecular complex between its CARD and FIIND domains (D’Ousualdo *et al.*, 2011; Jin, Huang *et al.*, 2013). These findings indicate that electrostatic interactions may play a role in mediating the association of the zIGBP1 CARD with its binding partners, either within the same protein, such as the GD or FIIND domains, or from other CARD-containing zebrafish proteins.

4. Conclusions

Here, we report the identification of a zebrafish protein zIGBP1 that contains guanylate-binding protein-like domains followed by a FIIND domain and a CARD. The FIIND–CARD domain arrangement is similar to the mammalian NLRP1 and CARD8 proteins, the FIIND domains of which were reported to undergo autocleavage as a regulatory mechanism for inflammasome activation. Interestingly, residues important for mammalian FIIND domain cleavage are also conserved in the zIGBP1 FIIND domain. The N-terminal GD and HD domains of zIGBP1 are typical of the mammalian guanylate-binding proteins, which indicates a potential function in antimicrobial immunity. The high-resolution crystal structure of the zIGBP1 CARD demonstrates a six-helix bundle fold similar to the NLRP1 CARD, with the bent $\alpha 1$ helix typical of all known CARD structures. The electrostatic surface of the zIGBP1 CARD suggests that it may associate with partner domains through charge–charge interactions similar to the type I interaction in the death-domain superfamily. Future studies will be required to identify its binding partner(s) and to characterize its physiological functions to definitely validate this domain as a *bona fide* CARD. Taken together, the current study lays a framework for future investigation of the structures of zebrafish CARD-containing proteins and their potential function in immune responses.

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