# *Did* cis- *and* trans-*defensins derive from a common ancestor*?

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## Immunogenetics

ISSN 0093-7711 Volume 71 Number 1

Immunogenetics (2019) 71:61-69 DOI 10.1007/s00251-018-1086-y





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#### **ORIGINAL ARTICLE**



# Did cis- and trans-defensins derive from a common ancestor?

Weiping Zhou<sup>1,2</sup> • Bin Gao<sup>1</sup> • Shunyi Zhu<sup>1</sup>

Received: 4 June 2018 / Accepted: 21 September 2018 / Published online: 2 October 2018 © Springer-Verlag GmbH Germany, part of Springer Nature 2018

#### Abstract

Defensins are small, cysteine-rich, cationic antimicrobial peptides, serving as effectors of the innate immune system and modulators of the adaptive immune system. They extensively exist in multicellular organisms and are divided into *cis* and *trans* according to their disulfide bridge connectivity patterns. It has been proposed that these two types of defensins convergently originated from different ancestors. Here, we report the discovery of a structural signature involved in the formation of the cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet (CS $\alpha\beta$ ) fold of the *cis*-defensins in some *trans*- $\beta$ -defensins, with only one amino acid indel (CXC vs. CC. C, cysteine; X, any amino acid). The indel of the X residue in the structural signature provides a possible explanation as to why *cis*- and *trans*-defensins possess different folds and connectivity patterns of disulfide bridges formed in evolution. Although our attempt to convert the structure type of a present-day *trans*-defensin with the X residue deleted was unsuccessful due to the low solubility of the synthetic peptide, a combination of data from structural signature, function, and phylogenetic distribution suggests that these defensins may have descended from a common ancestor. In this evolutionary scenario, we propose that a progenitor *cis*-scaffold might gradually evolve into a *trans*-defensin after deleting the X residue in specific lineages. This proposal adds a new dimension to more deeply studying the evolutionary relationship of defensins with different folds and of other distantly related proteins.

Keywords Antimicrobial peptide · Disulfide bridge · Structural signature · Fold change · Evolution

### Introduction

As one of the well-known groups of antimicrobial peptides, defensins are generally small, cysteine-rich, cationic peptides, which are present in a variety of multicellular organisms including invertebrates, vertebrates, fungi, and plants (Dias Rde and Franco 2015; Silva et al. 2014; Zasloff 2002). They are firstly named for the three human neutrophil peptides in 1985 (Ganz et al. 1985) and then the term is widely applied to numerous similar peptides. These molecules are divided into two superfamilies called *cis*-defensins and *trans*-defensins

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00251-018-1086-y) contains supplementary material, which is available to authorized users.

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based on the connectivity and orientation of their disulfide bridges (Shafee et al. 2016). The *cis*-defensins refer to a group of peptides that possess two parallel disulfide bridges connecting an  $\alpha$ -helix and a C-terminal  $\beta$ -strand. On the contrary, members of the *trans*-defensins encompass two disulfide bridges that orient in the opposite directions from the Cterminal  $\beta$ -strand binding to different secondary structure elements (Shafee et al. 2016, 2017). On the basis of this criterion, the cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet (CS $\alpha\beta$ ) defensins produced by plants, fungi, and invertebrates belong to *cis*defensins, whereas  $\alpha$ -defensins,  $\beta$ -defensins, and  $\theta$ defensins occurring in vertebrates as well as the big defensins from invertebrates (mollusks, arthropods, and chordates) are part of *trans*-defensins (Dias Rde and Franco 2015; Lehrer and Ganz 2002; Saito et al. 1995; Teng et al. 2012).

As the name reflects, these defensive peptides in the two superfamilies mainly possess antimicrobial activity for providing effective protection of the host against microbial infections before onset of diseases (Brogden 2005; Mattar et al. 2016; Silva et al. 2014). In addition to this property, some of them exert anticancer, toxic, signaling, and other effects (Shafee et al. 2017). Since the discovery of human  $\beta$ -

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defensin 2 (HBD-2) as an inducer of the chemoattraction of memory T cells and immature dendritic cells by interacting with CCR6, many studies have showed that  $\beta$ -defensins serve as modulators of inflammation and activators of linking the innate and adaptive immunity of vertebrates (Semple and Dorin 2012; Suarez-Carmona et al. 2015; Wu et al. 2003; Yang et al. 1999).

Although the structure and function of *cis*- and *trans*defensins have been extensively studied, their evolutionary link is still ambiguous due to highly variable sequences and structures. It has been proposed that the *cis*- and *trans*defensins are products of convergent evolution based on the orientation of the most conserved pair of disulfide bridges (Shafee et al. 2016). However, our recent research revealed fold change of homologous fungal defensin-like peptides in evolution via modification of their structural motifs (Wu et al. 2017). Moreover, in recent years accumulated evidences also suggest that protein structures could change during evolution by just few mutations in sequences (Cordes et al. 1999; Grishin 2001; He et al. 2012; Meier et al. 2007; Stewart et al. 2013). Hence, the possibility that *cis*- and *trans*defensins share a common ancestor cannot be ruled out.

To elucidate the evolutionary relationship of defensins, we firstly illuminated the structural signature of *cis*-defensins that is responsible for maintaining the  $CS\alpha\beta$  fold, and then performed database search to find some *trans*- $\beta$ -defensins containing this signature with only one amino acid deletion. This finding provides a theoretical foundation for different folded forms between *cis*- and *trans*-defensins and can be considered as a "molecular fossil" evidence to trace their evolutionary origin.

### Materials and methods

#### Sequence analysis

To illustrate the conserved structural signature of the CS $\alpha\beta$  fold present in *cis*-defensins, we searched for the functional *cis*-defensins from invertebrates and fungi in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/) (Wang et al. 2016) and established their regular expression pattern. Using the pattern, we performed new search against the UniProtKB Database (https://www.uniprot.org/) to find the eligible  $\beta$ -defensins. Sequences were aligned by CLUSTAL X program and further refined by hand with reference to the cysteine residue position. The distance of C $\alpha$  atoms between the glycine and the first X residue in the motif "CX(3)C" was calculated by Pymol (https://pymol.org/2/). The difference in the free energies ( $\Delta\Delta$ G, in kcal/mol) between the "mutant" and the "wild-type" structures of *cis*-defensins was calculated by FoldX (Schymkowitz et al. 2005).

#### Oxidative refolding of Gallinacin-11(CTC)

Gallinacin (Gal)-11 is a chicken  $\beta$ -defensin and Gal-11(CTC) is its mutant with a threonine insertion between the two cysteines in the "CC" motif. Its primary sequence is FSDSQLCRNNHGHCRRLCFHMESWAGSCMNGRLRC-TCR. Gal-11(CTC) was chemically synthesized in its reduced form by ChinaPeptides (Shanghai, China) with purity > 90%. For oxidative refolding, several different protocols were tried, including air oxidization in various alkaline solutions with or without DMSO or CuCl<sub>2</sub> and disulfide bridge shuffling with GSH/GSSG. Oxidized products were analyzed by reversed phase high-pressure liquid chromatography (RP-HPLC).

#### Analysis of phylogenetic distribution of defensins

A life tree containing major clades of multicellular organisms was constructed based on representative 18 s rRNA sequences using maximum-likelihood (ML) method by MEGA 6.0 (https://www.megasoftware.net/). One thousand bootstrap replicates were performed in the ML tree. The origin of representative 18 s rRNA sequences: Vertebrate (*Homo sapiens*: X03205), Cephalochordata (*Branchiostoma floridae*: M97571), Mollusca (*Mytilus galloprovincialis*: L33452), Arthropoda (*Belisarius xambeui*:AF005442; *Limulus polyphemus*: L81949), Nematoda (*Trefusia zostericola*: AF329937), Cnidaria (*Haloclava producta*: AF254379), and Fungi (*Saccharomyces cerevisiae*: NR\_132213).

#### Results

# *cis*-Defensins containing a conserved structural signature

As mentioned previously, *cis*-defensins adopt the  $CS\alpha\beta$  fold that comprises a single  $\alpha$ -helix spanning the "CX(3)C" motif and two antiparallel *β*-strands with the C-terminal *β*-strand covering the "CXC" motif connected to the  $\alpha$ -helix by two evolutionarily conserved disulfide bridges (Bontems et al. 1991; Zhu et al. 2005) (Fig. 1a). By analyzing the functional cis-defensins from fungi and invertebrates in the Antimicrobial Peptide Database (APD), we defined the regular sequence expression pattern of cis-defensins as C-X(5,16)-CX(3)C-X(7,9)-GXC-X(4,10)-CXC (X, any amino acid; C, cysteine) (Online Resource 1). Comparative structural analysis has showed that the framework "CX(3)C/CXC" could induce the formation of the CS $\alpha\beta$  fold and stabilize the structure (Tamaoki et al. 1998). And a highly similar distance between the two cysteine C $\alpha$  atoms in the two motifs "CX(3)C" and "CXC" provides a necessary condition for the formation of the two typical disulfide bridges (Wu et al. 2017) (Fig. 1b).

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**Fig. 1** The structural characteristics of the CSαβ fold. **a** Secondary structure topology of the CSαβ fold (cylinder, α-helix; arrow, β-strand). Seven evolutionarily conserved residues, including six cysteines and one glycine, are shown with reference to their positions. Gray dotted lines represent disulfide bridges. The picture is modified from Zhu et al. (2005). **b** The structural constraints of forming the CSαβ fold. The CSαβ fold is typified by the fungi defensin eurocin (PDB: 2LT8). Left: The distances between Cα atoms of cysteines in the motif "CX(3)C" or "CXC" is shown. The Cα atoms of cysteines are presented in yellow spheres. Right: The space limitation at intersection of the α-helix and the β-sheet is emphasized. The glycine and the first X

Additionally, the glycine in the motif "GXC" is the most common residue at the intersection of the  $\alpha$ -helix and the first  $\beta$ -strand. Structurally, the closest residue to the glycine is the first X residue in the "CX(3)C" motif. By analyzing the structures of 74 CS $\alpha\beta$  peptides from the Protein Data Bank (PDB) and calculating the distance of C $\alpha$  atoms between the glycine residue in the "CX(3)C" motif are presented in spheres and colored in green and magenta, respectively. **c** Mutation-induced changes in free energy of 74 CS $\alpha\beta$  peptides. For each peptide, the glycine in the "GXC" motif was mutated by other 19 amino acid residues and their free energy changes ( $\Delta G$ , predicted by FoldX) were compared with that of the wild-type peptide.  $\Delta\Delta G = \Delta G_{mutation} - \Delta G_{wildtype}$ , shown as average  $\pm$  standard deviation (SD) ( $\Delta\Delta G > 0$  indicates lower stability). "G" in the abscissa represents wild-type peptides with a GXC motif and other capital letters represent mutants with the glycine mutated by other residues

and the first X residue in the "CX(3)C" motif, we found that the glycine's side chain points to the  $\alpha$ -helix and the distance value is 4.41 ± 0.80 Å (Online Resource 2). As shown in Fig. 1b, the space between the glycine and the first X residue in the motif "CX(3)C" is too narrow to accommodate other residue's R-group. Furthermore, we calculated the free energy Author's personal copy

changes ( $\Delta\Delta G$ ) when the glycine in the "GXC" motif was mutated by other 19 amino acids using FoldX. The estimated changes in free energy are notably increased with regard to 72 native CS $\alpha\beta$  peptides with  $\Delta\Delta G$  values ranging from 4.43 and 59.47 kcal/mol (Fig. 1c, Online Resource 3), indicating structural instability when other residues are introduced at this position. This analysis well explains the cause of the glycine conservation and highlights its key structural role in stabilizing the proteins.

Based on these findings, we refine the structural signature of the  $CS\alpha\beta$  fold as "C...CX(3)C...GXC...CXC" where the six cysteines form three conserved disulfide bridges and the glycine deals with space limitation to commonly ensure the structural stability of this class of peptides.

# Some *trans*-β-defensins possessing the structural signature of *cis*-defensins with one amino acid deletion

Interestingly, using regular expression pattern searches we found that a total of 141  $\beta$ -defensins (135  $\beta$ -defensins from the UniProtKB and 6  $\beta$ -defensins from the reference (Tang et al. 2018)) possess a similar structural signature to that of *cis*-defensins (Fig. 2, Online Resource 4). The only difference is that  $\beta$ -defensins possess "CC" near the C-terminus while *cis*-defensins contain "CXC" in the same position (Fig. 2). It is reported that six cystines in  $\beta$ -defensins are also structurally conserved (Hoover et al. 2003; Krishnakumari et al. 2003; Wu et al. 2003; Yang et al. 2016). It thus is reasonable to infer that the X indel (CC vs. CXC) most likely results in different folds and connectivity patterns of disulfide bridges (*cis*-defensin:C1/C4, C2/C5, C3/C6;  $\beta$ -defensin:C1/C5, C2/C4, C3/C6) between *cis*-defensins and *trans*- $\beta$ -defensins, which prompted us to explore their structural correlation.

#### The structural relationship between *cis*and *trans*-defensins

As mentioned above, the framework "CX(3)C/CXC" leads to an  $\alpha$ -helix in the "C(X)3C" portion that is cross-linked to the "CXC" counterpart folded into an extended  $\beta$ -strand by disulfide bridges in a suitable distance (Tamaoki et al. 1998; Wu et al. 2017). The absence of the X residue definitely changes the distance of C $\alpha$  atoms between the two cysteines of the "CXC" motif. In this case, if the structure did not adjust accordingly, it would be impossible to form a stable fold in evolution. Based on the finding that some  $\beta$ -defensins possess the structural signature of the CS $\alpha\beta$  fold with only one X residue deletion in the "CXC" motif, we deduce that the *cis*defensins accidentally deleted the X residue in evolution and gradually accumulated some mutations for evolving into the  $\beta$ -defensin fold to protect themselves from environmental factors and to exert new functions. To further illustrate the structural relationship between the two types of defensins, we tried to testify the structural conversion from a *trans*- $\beta$ -defensin to a *cis*-defensin in laboratory. The existing chicken  $\beta$ -defensin Gal-11 was chosen to do experiment by sequence alignment. We chemically synthesized reduced Gal-11(CTC), a mutant of Gal-11 with a threonine insertion in the "CC" motif of Gal-11. Unfortunately, we found that this peptide possessed rather low water solubility and a few amount of soluble components did not oxidize into a single product at various oxidative-refolding conditions (Fig. S1), hampering further structural study. However, regardless of the experimental result, the structural signature of defensins described here provides new evidence for their evolutionary relationship.

#### Phylogenetic distribution of cis- and trans-defensins

Given that fungi and animals constitute a monophyletic group and defensins from these organisms are closely related (Zhu 2008), we construct a life tree to show the phylogenetic distribution of cis- and trans-defensins (Fig. 3). Up to now, the most ancient trans-defensins were found in sea anemones, belonging to the phylum Cnidaria, the oldest extant lineage of venomous animals (Torres and Kuchel 2004; Tysoe et al. 2016). By contrast, cis-defensins exhibit a more extensive distribution than trans-defensins since they have been found in fungi and even in plants (Carvalho Ade and Gomes 2009; Zhu 2008). In addition, the cis- and trans-defensins are both distributed in Arthropoda, Mollusca, and Cephalochordata. In vertebrates, there are only trans-defensins. Given that cisdefensins are distributed in fungi and animals (Opisthokonts) whereas trans-defensins can be only traced back to Planulozoa that is composed of the Cnidaria and the Bilateria (Fig. 3), it is clear that *cis*-defensins appeared earlier than trans-defensins, consistent with our above deduction.

## Discussion

According to structural characteristics of the CS $\alpha\beta$  fold, we propose that the *cis*- and *trans*-defensins are related structurally and the fold of *trans*-defensins might derive from an ancestral *cis*-defensin's scaffold. Although our experimental conversion between *cis*- and *trans*-defensins is unsuccessful in laboratory, this does not mean that a structural change did not occur in evolution. Firstly, as mentioned above, the  $\beta$ defensin fold might be gradually formed via accumulation of mutations after deleting the X residue in evolution. Secondly, defensins have already evolved hundreds of millions of years and their genes have been subjected to selection and rapid evolution, leading to enormous sequence diversity (Hughes 1999; Semple et al. 2006; Zou et al. 2007), making it difficult to find applicable surrogates of evolutionary intermediates M. musculus 091V82

M. musculus|Q91V70

C. l. familiaris O30KU3

C. l. familiaris|Q30KU5

C. l. familiaris|Q30KU6

A. fusciceps A8CYJ0

H. sapiens Q8WTQ1

H. sapiens|O8NET1

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							•			
Eurocin AP02119 2L18	GFG	PGD	-AYQ	SEH	RALGGGRTG	GYC	AGPWYLGHPI	Chi	SF	
Micasin1 AP02121 2LR5	GFG	PFN	-ENE <mark>C</mark>	CHAH	LSIGRKF	GFC	AGPLRA1		GKQ	
Plectasin AP00549 1ZFU	GFG	NGPWDEI	)DMQ <mark>C</mark>	CHNH <mark>C</mark> HNH	KSIKG-YKG	GYC	AKGGFV	CKC	Y	
MGD1 AP00438 1FJN	GFG	P-N	-NYQ <mark>C</mark>	HRH	KSIPG-RCG	GY	GGWHRLF	R <mark>C</mark> ⊡C	YRCG	
DefDM AP02593	GFG	PLN	-QGA <mark>C</mark>	CHNH <mark>C</mark> HNH	RSIRRRG	GYC	SGIIKQ1	CIC	YRN	
Dsdefensin AP02592	GFG	PLN	-QGA <mark>C</mark>	CHNH <mark>C</mark> HNH	RSIRRRG	GYC	SGIIKQ1	CIC	YRN	cis
HEdefensin AP02751	GFG	PLN	-QGA <mark>C</mark>	HRH	RSIRRRG	GYC	SGIIKQ1	CIC	YRN	-de
DefMT6 AP02591	GFG	PLN	-QGA	CHNHC	RSIKRRG	GYC	SGIIKQ1		YRK	fer
Longicin AP01468	GFG	PLN	-QGA	CHNH <mark>C</mark> HNH	RSIGRRG	GYC	AGIIKQ1	CIC	YRK	ısir
AcDEF AP00182	GFG	PLD	-QMQ	HRH	QTITG-RSG	GY	SGPLKL1	CIC	YR	IS
MytilinA AP00709	GFG	P-N	-DYP <mark>(</mark>	HRH	KSIPG-RAG	GY	GGAHRLF		YR	
MGD2 AP00711	GFG	P-N	-NYA	HQH	KSIRG-YCG	GY	AGWFRLF		YRCG	
AOD AP00539	GFG	PWN	RYQ	- HSH	RSI-G-RLG	GY	AGSLRL1	CIC	YRS	
DefMT7 AP02755	GFG	PKS	ALS	sqq	RENNT-HSG	GY	NGPFNI\	/ <mark>cs</mark> c	Y	
DefMT5 AP02590	GFF	PY	-NGY		RKKLR-RRG	GY	GGRWKL1		IMN	
C. livia R7VN63	LSDSQQ	RSN	-HGH		FHMERWE	GSC	SNGRLF	R <mark>C-</mark> C	R	1
Gal-11 Q6IV18-2	<u>FSDSQL</u>	RNN	-HGH	RRL	FHMESWA	GSC	MNGRLF	R <mark>C-</mark> C	<u>R</u>	
T. guttata A0A0U8TE77	DTQQ	RSS	-RGH	RRL	FHMERWE	GSC	SNGRLF	RC-C		
O. anatinus POC8A5	GMKEK	VTM	-GGY <mark>C</mark>	RKQ	RVQDALS	GY	RNENB	c-c	V	
O. anatinus P0C8A7	GISRVRI	REK	-GGH <mark>C</mark>		HLEERHL	GGC	RAAYLTB	c-c	2	
S. scrofa Q2VBJ9	ADRI	GYG	-TSR	RRY	KRQEIRI	GRC	PNTYB	c-c	LKKWR	
B. mutus L8I333	GFFDEK	YKL	-KGK	CIES C	QINEELI	GLC	QKSLŀ	( <mark>C-</mark> C	VALQPCGINKEGS	
R. norvegicus Q32ZI4	KKVYNAVS	MTN	GGI	WLK	SGTFREI	GSC	GTRQ-LF	( <mark>C-</mark> C	ККК	tra
R. norvegicus Q32ZH8	TLYRRFL	KKM	-KGR	ETA	LSFEKKI	GT	RADLTPI	c-c	КЕКККН	ns-
M. musculus Q9EPV9	KTINNPVS	CMI	-GGI <mark>C</mark>	RYL	KGNILQN	GSC	GVTS-LN	J <mark>C-</mark> C	KRK	÷
M. musculus Q91VD6	QLINSPVT	MSY	-GGS	QRS	NGGFRLG	GHC	GHPK-IF	c-c	RRK	def
M. musculus Q8R2I3	EIAVCET	RLG	RGK		IESEKIV	GWC	KLNFB	r <mark>c-</mark> c	RERI	iens
M. musculus Q8R2I6	-IIGVSEMER	нкк	-GGY	YFY	FSSHKKI	GSC	FPEW-PF	R <mark>C-</mark> C	KNIK	sing

GSP

NFR

RCLNGH-

CTKWRN

PNTY

LNSO

RCPNTY

RAELTP---I

Fig. 2 Multiple sequence alignment of *cis*- and *trans*- $\beta$ -defensins. The structural signature of cis-defensins, comprising six cysteines (shadowed in yellow) and one glycine (in green), is found in many trans-\beta-defensins. The presence or absence of an X residue in these defensins is shadowed in red. Secondary structure elements (cylinder,  $\alpha$ -helix; arrow,  $\beta$ -strand) and

-NEPVS

-NSKRA

NFDPKYRFER

--ARGREKET

IRN----GGI<mark>C</mark>OYR<mark>C</mark>IGLR--HKI

LQR

CIGLF--HKI

KTFCDDDE--YDY

QEF<mark>C</mark>LETE--IQA

YRE---GGE

AKV----KGI

CAHP---NGS

---AIHRRMQ<mark>C</mark>QKM----DGRCEVECLSFE--DKI

---EFELDRICGYG----TARCRKKCQNQE--YKIGI

-KFKEI<mark>C</mark>ERP---NGS<mark>C</mark>RDF<mark>C</mark>LETE--IHV<mark>G</mark>

---EFELDRI<mark>C</mark>GYG----TAR<mark>C</mark>RKK<mark>C</mark>RSOE--YRI

disulfide bridge connectivity patterns are shown at the top (cis) and bottom (trans) of the alignment, which are extracted from the structural coordinates of plectasin (PDB: 2LT8) and mBD7 (PDB: 1E4T), respectively. The amino acid sequence of Gal-11 for chemical synthesis is underlined twice

RPMVFESITEPTTPKE-

LRKWDESLLNRTKP---

LPLGHOPRIESTTPKKD

LKKWDESLLNFTKH

KKRKNN-

connecting the structural change of defensins and to reconstruct ancient sequences for experimental evolution. Therefore, it is understandable that we failed to get meaningful results from the experiment where an existing defensin was applied. However, the structural signature existed in both cisand *trans*-defensins highlights the possibility of structural conversion.

The antimicrobial activity is the most commonly reported function of defensins. Both cis-defensins in fungi and invertebrates and trans-defensins in vertebrates have a broadspectrum bactericidal property against Gram-positive and a

few Gram-negative bacteria. The cis-defensins in plants show activity primarily against fungi although some of them have been observed to inhibit the growth of Gram-positive and Gram-negative bacteria (Dias Rde and Franco 2015). Given that they both have retained antimicrobial activity during evolution, it is probable that antimicrobial activity is a common ancestral function. Importantly, some cis-defensins (e.g., plectasin (Schneider et al. 2010), eurocin (Oeemig et al. 2012), and Cg-Def (Schmitt et al. 2010) and trans-defensins (e.g., human  $\beta$ -defensin 3 (HBD-3) (Sass et al. 2010) and human  $\alpha$ -defensin 1 (HNP-1) (De Leeuw et al. 2010)) are Fig. 3 The simplified phylogeny of fungi and animals used to annotate the occurrence of different families of defensins in different lineages. "+" means presence and "--" means absence. Note: In Cnidaria peptides with a  $\beta$ -defensin fold are toxins or  $\alpha$ amylase inhibitors (Torres and Kuchel 2004; Tysoe et al. 2016)

# $CS\alpha\beta | Big | \alpha | \beta | \theta Defensin$



reported to employ a common mechanism in bacterial killing, where the cell wall precursor lipid II is targeted to inhibit the cell wall biosynthesis. This provides further evidence for their common origin.

We also think about the reason why the  $CS\alpha\beta$  fold could evolve into a β-defensin fold. Previous studies have showed that disulfide bridges play an important role in functional performance of the peptides with a  $CS\alpha\beta$  fold (Sun et al. 2002; Yamaguchi et al. 2016). In contrast, the disulfide bridges in some β-defensins although required for binding and activation of receptors for chemotaxis, are dispensable for their antibacterial function (Schroeder et al. 2011; Wu et al. 2003). In other words, the bactericidal activity of these  $\beta$ -defensins is structure-independent. In general, a functional gene will lose its function by deleterious mutations that destroy its protein structure and function. These deleterious mutations are usually eliminated by purifying selection, and thus there is no fold change in evolution (Fig. 4a). However, for an ancestral cisdefensin with the X residue deletion, it may be an exception since its antibacterial function could still be remained even though its structure is destroyed due to the deletion (Fig. 4b). Hence, as the prototype of  $\beta$ -defensins, these structure-deficient cis-defensins are preserved in genomes and function as antimicrobial agents. With the emergence of the adaptive immunity in the vertebrate lineage, these archetypal  $\beta$ -defensing finally evolved into  $\beta$ -defensing with novel functional properties (i.e., activators of receptors for chemotaxis). Like chemokines, a defined disulfide-stabilized 3D structure is also required for correct binding of  $\beta$ -defensins to their receptors (Wu et al. 2003) (Fig. S2), we thus assume that the chemotactic interaction might be a force driving the evolution of  $\beta$ -defensin fold in the vertebrate lineage. Because it has been proposed that invertebrate defensins might have a chemotactic activity, as do their mammalian counterparts (Boulanger et al. 2006), it is possible that the evolution of *trans*-defensins from *cis*-defensins might have occurred earlier, as evidenced by the presence of *trans*-defensins (big defensins) in some invertebrate lineages.

Previous studies have showed that vertebrate transdefensins share a common ancestry and have undergone evolution from  $\beta$ -defensins to  $\alpha$ -defensins to  $\theta$ -defensins (Li et al. 2014; Liu et al. 1997; Tang et al. 1999) (Fig. 3). Besides, big defensins have been considered as the ancestor of  $\beta$ -defensing based on the 3D structure and genomic data (Zhu and Gao 2013). Meanwhile, some studies about evolution of  $CS\alpha\beta$ -defensins suggested that defensins in invertebrates and fungi descended from a common ancestry that could be traced to myxobacterial defensin-like peptides (Tassanakajon et al. 2015; Zhu 2007; Zhu 2008). However, the diversity in genomic organization, sequences, and 3D structures hinders the establishment of the evolutionary relationship between cis- and trans-defensins. A phylogenetic study showed that there is a closer relationship between vertebrate  $\beta$ -defensins and insect defensins than between vertebrate  $\alpha$ - and  $\beta$ -defensions, but it is difficult to establish the ancestral state of structural characters (Hughes 1999). In this study, comparison of structural motifs gives a new clue to infer their evolutionary relationship. In addition, although structural change is slower than sequence do, there are evidences in recent years about protein fold change during the course of evolution (Ingles-Prieto et al. 2013). Insertion or deletion of one or a few amino acids within regular secondary structure



elements is probable to interrupt backbone topology and then a new protein fold evolve from an existing fold by the accumulation of simple substitution mutations (Cordes et al. 1999; Stewart et al. 2013). For example, the proteins Xfaso1 and Pfl6 from the Cro family that share a common ancestor have different folds. Therefore, structural dissimilarity does not mean no evolutionary relationship in view of fold change of proteins in evolution.

In summary, in this work, we propose for the first time that the *cis*- and *trans*-defensins possess similar structural signature and suggest that they might originate from a common ancestor via an amino acid deletion mutation in the structural motif. It appears that evolutionary retention of an original function (here antibacterial activity) in a mutant with the structure destroyed is a prerequisite for evolution of a new fold type (Fig. 4). It is expected that further study will help uncover the "real" relationship between these defensins. This will contribute to a better understanding of the evolutionary process of immune molecules between invertebrates and vertebrates and will also promote the development of comparative immunology among remote species.

Author's contributions S.Z. conceived and designed the research. W.Z. performed sequence and structural analyses. G.B. performed oxidative refolding experiments of peptides. W.Z., B.G., and S.Z. jointly wrote the paper.

**Funding** This work was supported by the National Natural Science Foundation of China (Grant Nos. 31870766 and 31570773) to S.Z.

#### **Compliance with ethical standards**

**Conflicts of interest** The authors declare that they have no conflict of interest.

#### References

- Bontems F, Roumestand C, Gilquin B, Menez A, Toma F (1991) Refined structure of charybdotoxin: common motifs in scorpion toxins and insect defensins. Science 254:1521–1523
- Boulanger N, Bulet P, Lowenberger C (2006) Antimicrobial peptides in the interactions between insects and flagellate parasites. Trends Parasitol 22:262–268
- Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. https://doi.org/ 10.1038/nrmicro1098
- Carvalho Ade O, Gomes VM (2009) Plant defensins–prospects for the biological functions and biotechnological properties. Peptides 30: 1007–1020. https://doi.org/10.1016/j.peptides.2009.01.018
- Cordes MH, Walsh NP, McKnight CJ, Sauer RT (1999) Evolution of a protein fold *in vitro*. Science 284:325–328
- De Leeuw E et al (2010) Functional interaction of human neutrophil peptide-1 with the cell wall precursor lipid II. FEBS Lett 584: 1543–1548. https://doi.org/10.1016/j.febslet.2010.03.004
- Dias Rde O, Franco OL (2015) Cysteine-stabilized alphabeta defensins: from a common fold to antibacterial activity. Peptides 72:64–72. https://doi.org/10.1016/j.peptides.2015.04.017
- Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, Lehrer RI (1985) Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 76:1427–1435. https://doi.org/10.1172/ JCI112120
- Grishin NV (2001) Fold change in evolution of protein structures. J Struct Biol 134:167–185. https://doi.org/10.1006/jsbi.2001.4335

- He Y, Chen Y, Alexander PA, Bryan PN, Orban J (2012) Mutational tipping points for switching protein folds and functions. Structure 20:283–291. https://doi.org/10.1016/j.str.2011.11.018
- Hoover DM, Wu Z, Tucker K, Lu W, Lubkowski J (2003) Antimicrobial characterization of human beta-defensin 3 derivatives. Antimicrob Agents Chemother 47:2804–2809
- Hughes AL (1999) Evolutionary diversification of the mammalian defensins. Cell Mol Life Sci 56:94–103
- Ingles-Prieto A, Ibarra-Molero B, Delgado-Delgado A, Perez-Jimenez R, Fernandez JM, Gaucher EA, Sanchez-Ruiz JM, Gavira JA (2013) Conservation of protein structure over four billion years. Structure 21:1690–1697. https://doi.org/10.1016/j.str.2013.06.020
- Krishnakumari V, Sharadadevi A, Singh S, Nagaraj R (2003) Single disulfide and linear analogues corresponding to the carboxyterminal segment of bovine beta-defensin-2: effects of introducing the beta-hairpin nucleating sequence d-pro-gly on antibacterial activity and biophysical properties. Biochemistry 42:9307–9315. https://doi.org/10.1021/bi034403y
- Lehrer RI, Ganz T (2002) Defensins of vertebrate animals. Curr Opin Immunol 14:96–102
- Li D, Zhang L, Yin H, Xu H, Trask JS, Smith DG, Li Y, Yang M, Zhu Q (2014) Evolution of primate alpha and theta defensins revealed by analysis of genomes. Mol Biol Rep 41:3859–3866. https://doi.org/ 10.1007/s11033-014-3253-z
- Liu L, Zhao C, Heng HH, Ganz T (1997) The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. Genomics 43:316–320. https://doi.org/10.1006/geno.1997.4801
- Mattar EH, Almehdar HA, Yacoub HA, Uversky VN, Redwan EM (2016) Antimicrobial potentials and structural disorder of human and animal defensins. Cytokine Growth Factor Rev 28:95–111. https://doi.org/10.1016/j.cytogfr.2015.11.002
- Meier S, Jensen PR, David CN, Chapman J, Holstein TW, Grzesiek S, Ozbek S (2007) Continuous molecular evolution of protein-domain structures by single amino acid changes. Curr Biol 17:173–178. https://doi.org/10.1016/j.cub.2006.10.063
- Oeemig JS, Lynggaard C, Knudsen DH, Hansen FT, Nørgaard KD, Schneider T, Vad BS, Sandvang DH, Nielsen LA, Neve S, Kristensen HH, Sahl HG, Otzen DE, Wimmer R (2012) Eurocin, a new fungal defensin: structure, lipid binding, and its mode of action. J Biol Chem 287:42361–42372. https://doi.org/10.1074/jbc.M112. 382028
- Saito T, Kawabata SI, Shigenaga T, Takayenoki Y, Cho J, Nakajima H, Hirata M, Iwanaga S (1995) A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity. J Biochem 117:1131–1137
- Sass V, Schneider T, Wilmes M, Korner C, Tossi A, Novikova N, Shamova O, Sahl HG (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in staphylococci. Infect Immun 78:2793–2800. https://doi.org/10.1128/iai.00688-09
- Schmitt P, Wilmes M, Pugnière M, Aumelas A, Bachère E, Sahl HG, Schneider T, Destoumieux-Garzón D (2010) Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. J Biol Chem 285: 29208–29216. https://doi.org/10.1074/jbc.M110.143388
- Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK, Mygind PH, Raventos DS, Neve S, Ravn B, Bonvin AMJJ, de Maria L, Andersen AS, Gammelgaard LK, Sahl HG, Kristensen HH (2010) Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. Science 328:1168–1172. https://doi.org/10.1126/science.1185723
- Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J (2011) Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1. Nature 469:419–423. https://doi.org/10.1038/ nature09674

- Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Serrano L (2005) The FoldX web server: an online force field. Nucleic Acids Res 33: W382–W388. https://doi.org/10.1093/nar/gki387
- Semple F, Dorin JR (2012) Beta-defensins: multifunctional modulators of infection, inflammation and more? J Innate Immun 4:337–348. https://doi.org/10.1159/000336619
- Semple CA, Gautier P, Taylor K, Dorin JR (2006) The changing of the guard: molecular diversity and rapid evolution of beta-defensins. Mol Divers 10:575–584. https://doi.org/10.1007/s11030-006-9031-7
- Shafee TM, Lay FT, Hulett MD, Anderson MA (2016) The defensins consist of two independent, convergent protein Superfamilies. Mol Biol Evol 33:2345–2356. https://doi.org/10.1093/molbev/msw106
- Shafee TM, Lay FT, Phan TK, Anderson MA, Hulett MD (2017) Convergent evolution of defensin sequence, structure and function. Cell Mol Life Sci 74:663–682. https://doi.org/10.1007/s00018-016-2344-5
- Silva PM, Goncalves S, Santos NC (2014) Defensins: antifungal lessons from eukaryotes. Front Microbiol 5:97. https://doi.org/10.3389/ fmicb.2014.00097
- Stewart KL, Nelson MR, Eaton KV, Anderson WJ, Cordes MH (2013) A role for indels in the evolution of Cro protein folds. Proteins 81: 1988–1996. https://doi.org/10.1002/prot.24358
- Suarez-Carmona M, Hubert P, Delvenne P, Herfs M (2015) Defensins: "simple" antimicrobial peptides or broad-spectrum molecules? Cytokine Growth Factor Rev 26:361–370. https://doi.org/10.1016/ j.cytogfr.2014.12.005
- Sun YM, Liu W, Zhu RH, Goudet C, Tytgat J, Wang DC (2002) Roles of disulfide bridges in scorpion toxin BmK M1 analyzed by mutagenesis. J Pept Res 60:247–256
- Tamaoki H, Miura R, Kusunoki M, Kyogoku Y, Kobayashi Y, Moroder L (1998) Folding motifs induced and stabilized by distinct cystine frameworks. Protein Eng 11:649–659
- Tang KY, Wang X, Wan QH, Fang SG (2018) A crucial role of paralogous beta-defensin genes in the Chinese alligator innate immune system revealed by the first determination of a Crocodilia defensin cluster. Dev Comp Immunol 81:193–203. https://doi.org/ 10.1016/j.dci.2017.11.018
- Tang YQ, Yuan J, Osapay G, Osapay K, Tran D, Miller CJ, Ouellette AJ, Selsted ME (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science 286:498–502
- Tassanakajon A, Somboonwiwat K, Amparyup P (2015) Sequence diversity and evolution of antimicrobial peptides in invertebrates. Dev Comp Immunol 48:324–341. https://doi.org/10.1016/j.dci.2014.05. 020
- Teng L, Gao B, Zhang S (2012) The first chordate big defensin: identification, expression and bioactivity. Fish Shellfish Immunol 32:572– 577. https://doi.org/10.1016/j.fsi.2012.01.007
- Torres AM, Kuchel PW (2004) The beta-defensin-fold family of polypeptides. Toxicon 44:581–588. https://doi.org/10.1016/j.toxicon. 2004.07.011
- Tysoe C, Williams LK, Keyzers R, Nguyen NT, Tarling C, Wicki J, Goddard-Borger ED, Aguda AH, Perry S, Foster LJ, Andersen RJ, Brayer GD, Withers SG (2016) Potent human alpha-amylase inhibition by the beta-defensin-like protein helianthamide. ACS Cent Sci 2:154–161. https://doi.org/10.1021/acscentsci.5b00399
- Wang G, Li X, Wang Z (2016) APD3: the antimicrobial peptide database as a tool for research and education. Nucleic Acids Res 44:D1087– D1093. https://doi.org/10.1093/nar/gkv1278
- Wu Y, Gao B, Zhu S (2017) New fungal defensin-like peptides provide evidence for fold change of proteins in evolution. Biosci Rep 37: BSR20160438. https://doi.org/10.1042/BSR20160438
- Wu Z, Hoover DM, Yang D, Boulegue C, Santamaria F, Oppenheim JJ, Lubkowski J, Lu W (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3.

Proc Natl Acad Sci U S A 100:8880-8885. https://doi.org/10.1073/ pnas.1533186100

- Yamaguchi Y, Peigneur S, Liu J, Uemura S, Nose T, Nirthanan S, Gopalakrishnakone P, Tytgat J, Sato K (2016) Role of individual disulfide bridges in the conformation and activity of spinoxin (alpha-KTx6.13), a potassium channel toxin from Heterometrus spinifer scorpion venom. Toxicon 122:31–38. https://doi.org/10. 1016/j.toxicon.2016.09.013
- Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schröder JM, Wang JM, Howard OM, Oppenheim JJ (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286:525–528
- Yang M, Zhang C, Zhang X, Zhang MZ, Rottinghaus GE, Zhang S (2016) Structure-function analysis of avian beta-defensin-6 and beta-defensin-12: role of charge and disulfide bridges. BMC Microbiol 16:210. https://doi.org/10.1186/s12866-016-0828-y
- Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395. https://doi.org/10.1038/415389a

- Zhu S (2007) Evidence for myxobacterial origin of eukaryotic defensins. Immunogenetics 59:949–954. https://doi.org/10.1007/s00251-007-0259-x
- Zhu S (2008) Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of the CSalphabeta defensins. Mol Immunol 45:828–838. https://doi.org/10.1016/j. molimm.2007.06.354
- Zhu S, Gao B (2013) Evolutionary origin of beta-defensins. Dev Comp Immunol 39:79–84. https://doi.org/10.1016/j.dci.2012.02.011
- Zhu S, Gao B, Tytgat J (2005) Phylogenetic distribution, functional epitopes and evolution of the CSalphabeta superfamily. Cell Mol Life Sci 62:2257–2269. https://doi.org/10.1007/s00018-005-5200-6
- Zou J, Mercier C, Koussounadis A, Secombes C (2007) Discovery of multiple beta-defensin like homologues in teleost fish. Mol Immunol 44:638–647. https://doi.org/10.1016/j.molimm.2006.01. 012