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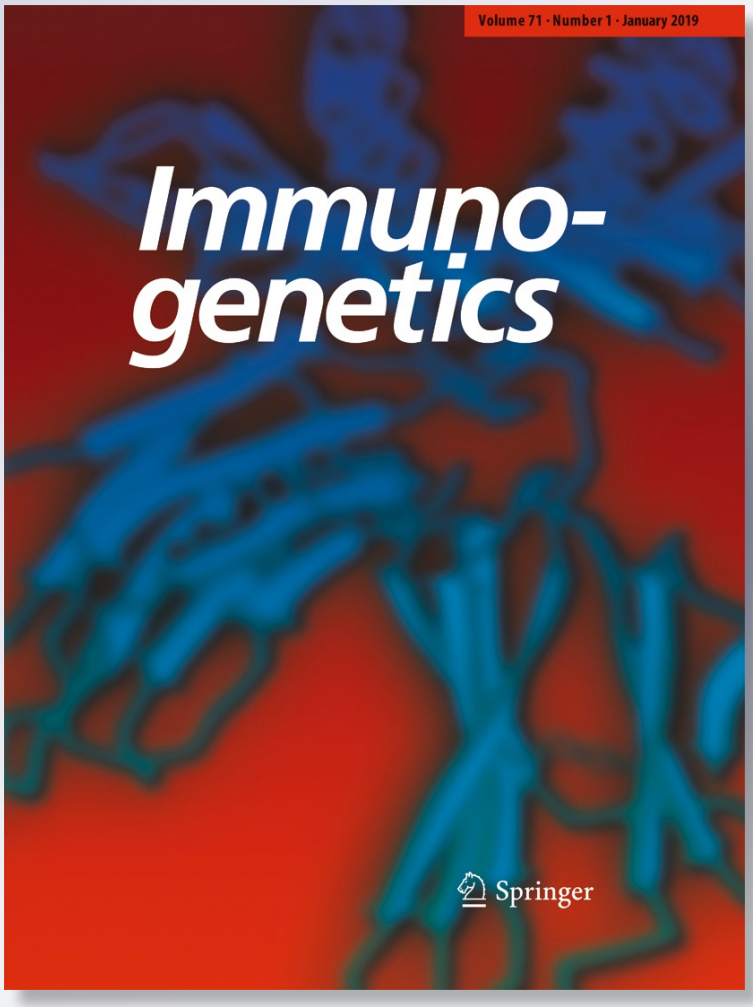

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ORIGINAL ARTICLE

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

Weiping Zhou[1,2] · Bin Gao[1] · Shunyi Zhu[1]

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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 α-helix/β-sheet (CSαβ) fold of the *cis*-defensins in some *trans*-β-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 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 α-helix and a C-terminal β-strand. On the contrary, members of the *trans*-defensins encompass two disulfide bridges that orient in the opposite directions from the C-terminal β-strand binding to different secondary structure elements (Shafee et al. 2016, 2017). On the basis of this criterion, the cysteine-stabilized α-helix/β-sheet (CSαβ) defensins produced by plants, fungi, and invertebrates belong to *cis*-defensins, whereas α-defensins, β-defensins, and θ-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 β-

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✉ Shunyi Zhu
zhusy@ioz.ac.cn

[1] Group of Peptide Biology and Evolution, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

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 β-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 CSαβ fold, and then performed database search to find some *trans*-β-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 CSαβ 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 β-defensins. Sequences were aligned by CLUSTAL X program and further refined by hand with reference to the cysteine residue position. The distance of Cα 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 (ΔΔG, 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 β-defensin and Gal-11(CTC) is its mutant with a threonine insertion between the two cysteines in the "CC" motif. Its primary sequence is FSDSQLCRNNHGHCRRLCFHMESWAGSCMNGRLRCTCR. 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 $CuCl_2$ 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 CSαβ fold that comprises a single α-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 α-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 CSαβ fold and stabilize the structure (Tamaoki et al. 1998). And a highly similar distance between the two cysteine Cα 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).

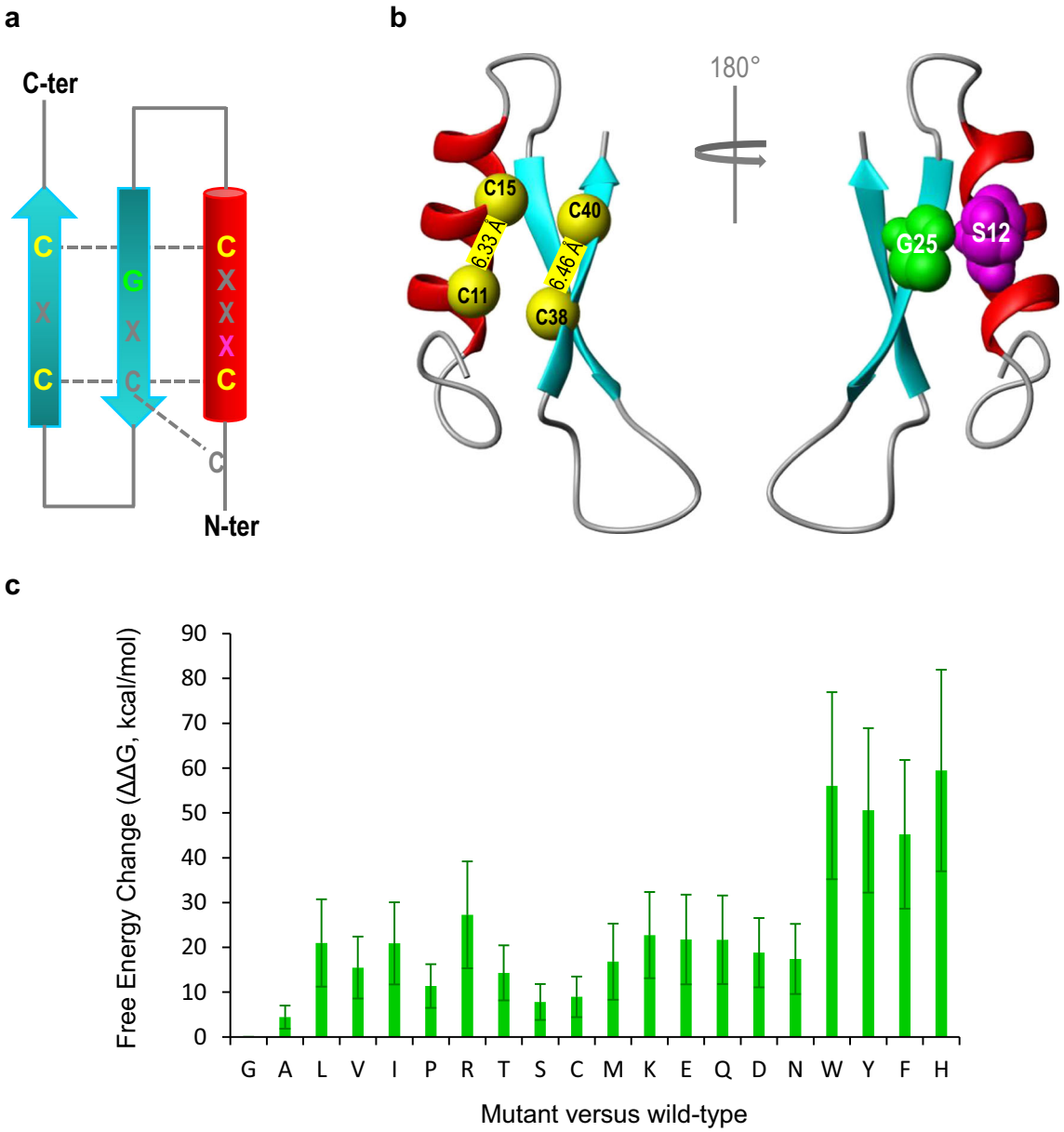

Fig. 1 The structural characteristics of the CSαβ fold. **a** Secondary structure topology of the CSαβ fold (cylinder, α-helix; arrow, β-strand). Seven evolutionarily conserved residues, including six cysteines and one glycine, are shown with reference to their positions. Gray dotted lines represent 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 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 CSαβ peptides. For each peptide, the glycine in the "GXC" motif was mutated by other 19 amino acid residues and their free energy changes (ΔG, predicted by FoldX) were compared with that of the wild-type peptide. $\Delta\Delta G = \Delta G_{mutation} - \Delta G_{wildtype}$, shown as average ± 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

Additionally, the glycine in the motif "GXC" is the most common residue at the intersection of the α-helix and the first β-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 CSαβ peptides from the Protein Data Bank (PDB) and calculating the distance of Cα atoms between the glycine and the first X residue in the "CX(3)C" motif, we found that the glycine's side chain points to the α-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

changes (ΔΔG) 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 CSαβ peptides with ΔΔG 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 CSαβ 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 β-defensins (135 β-defensins from the UniProtKB and 6 β-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 β-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 β-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; β-defensin:C1/C5, C2/C4, C3/C6) between *cis*-defensins and *trans*-β-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 α-helix in the "C(X)3C" portion that is cross-linked to the "CXC" counterpart folded into an extended β-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 Cα 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 β-defensins possess the structural signature of the CSαβ 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 β-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*-β-defensin to a *cis*-defensin in laboratory. The existing chicken β-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 *cis*-defensins 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 CSαβ 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 β-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

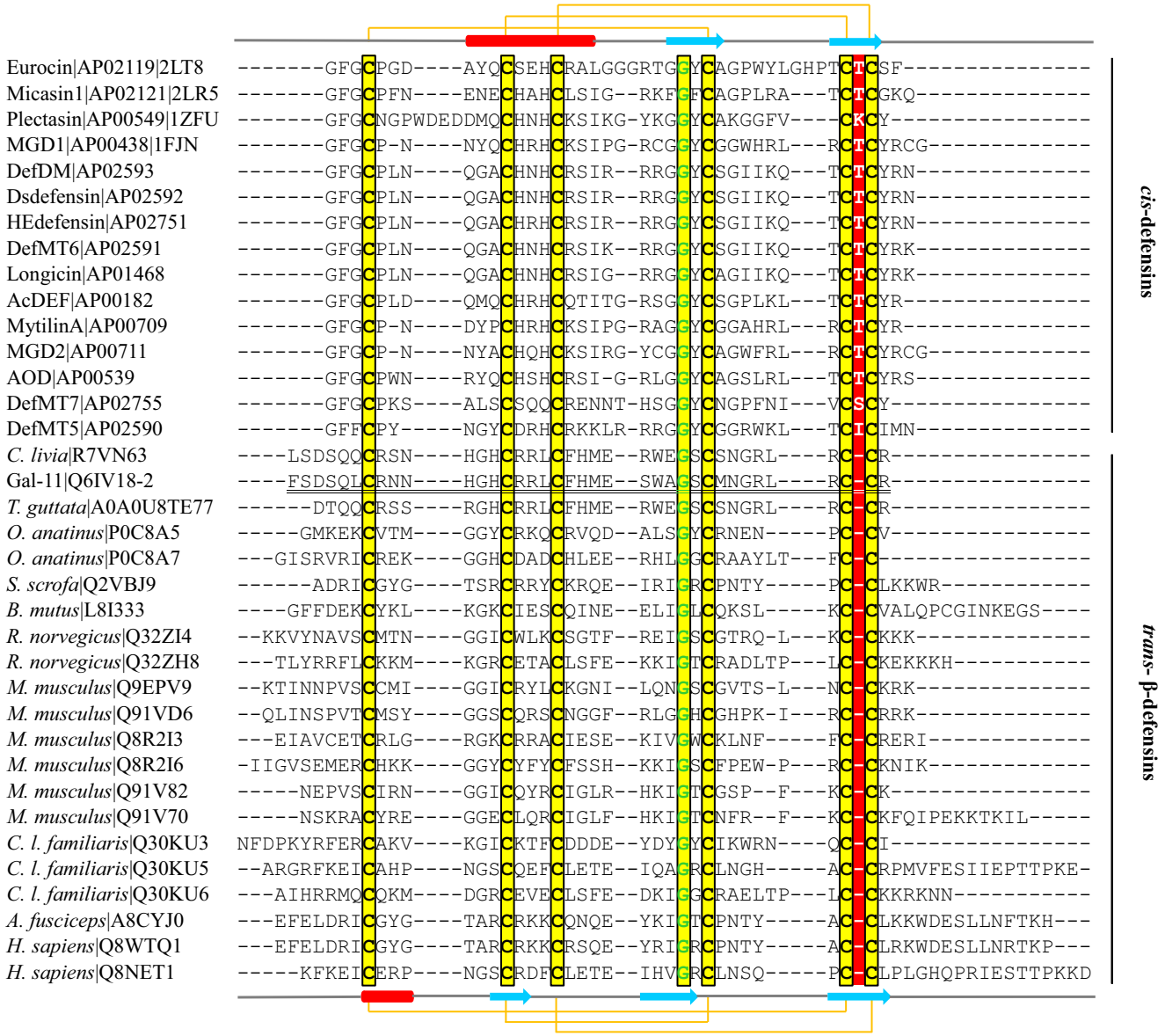

Fig. 2 Multiple sequence alignment of *cis*- and *trans*-β-defensins. The structural signature of *cis*-defensins, comprising six cysteines (shadowed in yellow) and one glycine (in green), is found in many *trans*-β-defensins. The presence or absence of an X residue in these defensins is shadowed in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

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 *cis*- and *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 broad-spectrum 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 β-defensin 3 (HBD-3) (Sass et al. 2010) and human α-defensin 1 (HNP-1) (De Leeuw et al. 2010)) are

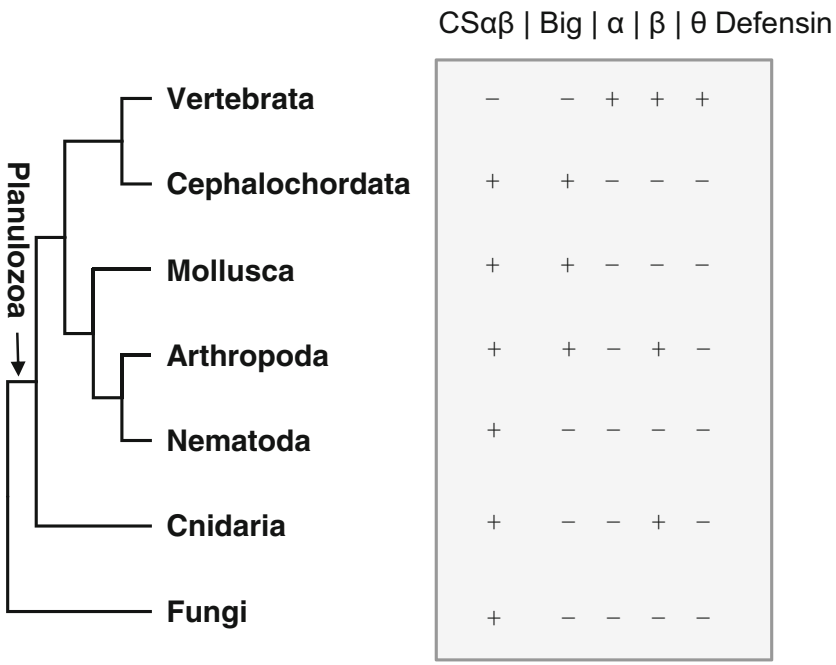

Fig. 3 The simplified phylogeny of fungi and animals used to annotate the occurrence of different families of defensins in different lineages. "+" means presence and "−" means absence. Note: In Cnidaria peptides with a β-defensin fold are toxins or α-amylase inhibitors (Torres and Kuchel 2004; Tysoe et al. 2016)

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 CSαβ 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 CSαβ 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 β-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 *cis*-defensin 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 β-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 β-defensins finally evolved into β-defensins 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 β-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 β-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 *trans*-defensins share a common ancestry and have undergone evolution from β-defensins to α-defensins to θ-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 β-defensins based on the 3D structure and genomic data (Zhu and Gao 2013). Meanwhile, some studies about evolution of CSαβ-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 β-defensins and insect defensins than between vertebrate α- and β-defensins, 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

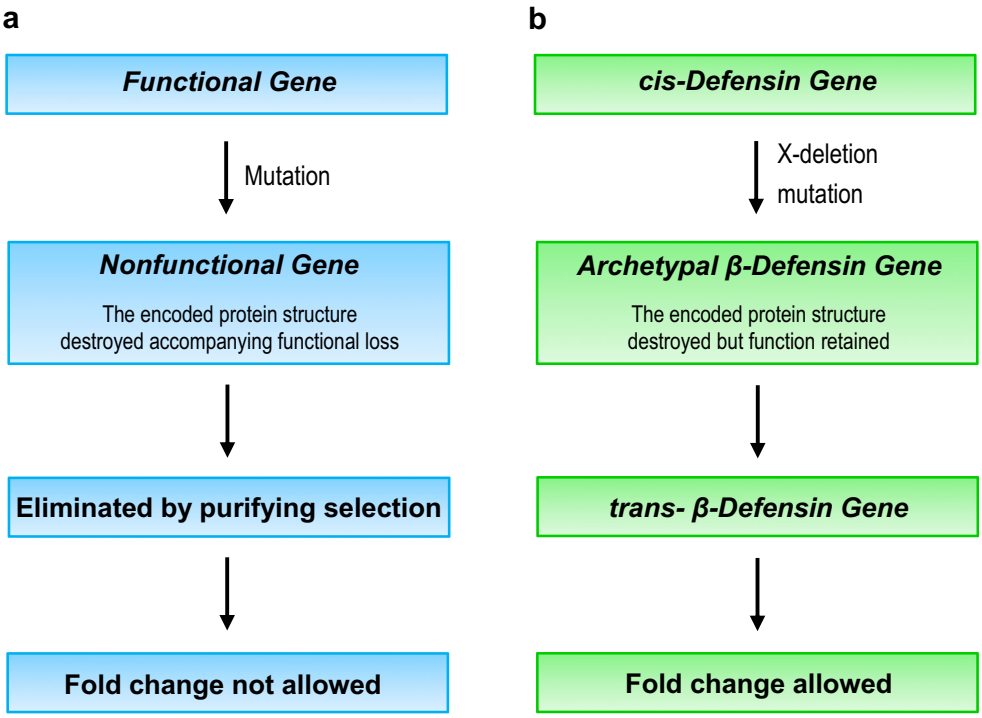

Fig. 4 A proposed scenario for mutation-mediated protein fold evolution. **a**. The generally accepted view that deleterious mutations destroying both structure and function of a functional gene-encoded protein will be eliminated by purifying selection, leading to no fold change occurrence in evolution; **b**. Mutation-mediated defensin fold change. In this process, the antibacterial function of an old *cis*-defensin with the X-residue deleted and structure destroyed might still be remained and acted as an archetypal β-defensin with this activity. Finally, this prototype evolved into *trans*-β-defensins with novel functional properties

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.

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Compliance with ethical standards

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

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