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Evidence for myxobacterial origin of eukaryotic defensins

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Abstract Antimicrobial defensins with the cysteine-stabilized α-helical and β-sheet (CSαβ) motif are a large family of ancient, evolutionarily related innate immunity effectors of multicellular organisms. Although the widespread distribution in plants, fungi, and invertebrates suggests their uniqueness to Eukarya, it is unknown whether these eukaryotic defensins originated before or posterior to the emergence of eukaryotes. In this study, we provide evidence in support of the existence of defensin-like peptides (DLPs) in myxobacteria based on structural bioinformatics analysis, which recognized two bacterial peptides with a conserved cysteine-stabilized α-helical motif, a nested structural unit of the CSαβ motif. Similarity in sequence and structure to fungal DLPs together with restricted distribution to the myxobacteria as well as central role of the myxobacteria in the origin of eukaryotes suggest that the bacterial DLPs represent the ancestor of the eukaryotic defensins and could mediate immune defense of early eukaryotes after gene transfer to the proto-eukaryotic genome. Our work thus offers a basis for further investigation of prokaryotic origin of eukaryotic immune effector molecules.

Keywords Defensin · The syntrophic hypothesis · Comparative modeling · Innate immunity

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Introduction

The CSαβ-type defensins are a large family of pivotal effector elements of innate immunity against microbial infection in multicellular organisms (Dimarcq et al. 1998). Their protective roles have been well documented by in vivo targeted disruption of the mosquito *Anopheles gambiae defensin* gene causing the death of the mosquitoes after Gram-positive bacterial infection (Blandin et al. 2002). All members of it possess a conserved structural motif comprising one α-helix and one β-sheet of two antiparallel strands. The helix spanning the CXXXC sequence (X, any amino acid) is connected to the second β-strand containing the CXC sequence via two disulfide bridges. The third disulfide bridge links the N terminus to the first β-strand (Cornet et al. 1995). Although exon shuffling and convergent evolution have proposed to explain the evolutionary history of this diverse family of peptides (Froy and Gurevitz 2003; Froy 2005), such structural conservation together with functional relatedness provides convincing evidence for their origin from a common ancestor (also see Rodriguez and Possani 2005).

Since the first representatives were isolated from two dipteran species (the flesh fly *Sarcophaga peregrine* and the black blowfly *Phormia terranovae*), more than 70 different defensins have been identified from some phylogenetic distant invertebrates, such as insects, crustaceans, ticks, spiders, scorpions, and mollusks (Bulet et al. 2004). Subsequently, defensins with a similar folding to the invertebrate defensins have also been found in most plants (Lay and Anderson 2005). More recently, a defensin-like peptide (named plectasin) has been characterized from a saprophytic fungus, which shows a high degree of sequence and structural similarity to the ancient invertebrate-type defensins (Mygind et al. 2005). High effectiveness against

the bacteria *Streptococcus pneumoniae* and *Streptococcus pyogenes* makes it an attractive candidate for the development of new type therapeutic drugs. After this discovery, we took advantage of computational approaches identifying additional 25 new fungal DLP genes, which form six distinct defensin families (Zhu 2008). The widespread distribution of the DLPs in most plants, fungi (Ascomycota and Zygomycota), and various invertebrates suggests their uniqueness to Eukarya. However, it is unknown whether they originated before or posterior to the emergence of eukaryotes. In this study, we identified two prokaryotic peptides from myxobacteria that show detectable sequence similarity to the eukaryotic DLPs from the fungus *Rhizopus oryzae*, a species near the base of the kingdom fungi phylogeny. Furthermore, comparative modeling-based structural analysis confirms that these bacterial DLPs can adopt a typical defensin fold. This provides key structural evidence for elucidation of the origin of eukaryotic defensins.

Materials and methods

Database search

BLASTP and TBLASTN programs were used to find possible prokaryotic defensin-like peptides from the microbial database under the default parameters (http://www.ncbi.nlm.nih.gov) using amino acid sequences of representative defensin precursors from plants, fungi, and animals as queries. TBLASTN compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands) using the BLAST algorithm (Altschul et al. 1990). This feature makes TBLASTN especially suitable for the discovery of remote homologues with a conserved sequence motif. The strategy used here has succeeded in finding of 25 new defensin-like peptides from model fungal genomes. Detailed methods used here have been described previously (Zhu 2008).

Fold recognition and comparative modeling

Fold compatibility between the bacterial defensin-like peptides AdDLP and SaDLP and known 3D structures was performed through GenTHREADER (Bryson et al. 2005). The plectasin structure (pdb entry 1ZFU) was selected as the template for modeling of AdDLP and its mutant (AdDLP3SS) where two cysteines were added. Standard comparative modeling approaches are used here for structural analysis (Duret et al. 1998; Cohen-Gonsaud et al. 2004). For constructing structural models of AdDLP and AdDLP3SS, sequence alignment was undertaken using the CLUSTAL X program and further refined by hand to remove gaps within α-helical and β-strand regions. Once the accurate alignment was determined, 3D models were generated with programs TITO and MODELLER. Models were evaluated by the Verify3D (http://bioserv.cbs.cnrs.fr/). Structural superimposition and root mean square deviation (RMSD) calculation were performed using Swiss-PdbViewer program (http://swissmodel.expasy.org/spdbv). The 3D protein models of AdDLP and AdDLP3SS have been submitted to the Protein Model database (Castrignanò et al. 2006) under accession numbers of PM0074723 and PM0074954, respectively.

Results

To trace the possible prokaryotic ancestor of the eukaryotic CSαβ defensins, we performed BLAST searches of the microbial genome database using representatives of the defensins from three eukaryotic kingdoms as queries (Dimarcq et al. 1998; Bulet et al. 2004; Lay and Anderson 2005; Mygind et al. 2005; Zhu 2008). These searches detected only two myxobacterial sequences from *Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca* similar to DLPs (Rorsin-1 and Rorsin-2) from the fungus *R. oryzae* (Zhu 2008; Fig. 1a). These two proteins represent precursors identified by the existence of typical signal sequences in their N termini and propeptide-like sequences in their C termini with typical basic amino acid cleavage signals (Fig. 1b). Previous studies have identified several types of precursor organization of CSαβ-type defensins differing in propeptide location. In this aspect, the precursor organization of bacterial DLPs more resembles the defensins from nematodes and mollusks (Zhang and Kato 2003; Fig. 1b). The predicted mature peptides, referred herein to as AdDLP (*A. dehalogenans* defensin-like peptide) and SaDLP (*S. aurantiaca* defensin-like peptide), have a compatible size to Rorsins. Thirty-one percentage sequence identity and 56 sequence similarity can be found between AdDLP and Rorsin-1. Among the 16 conserved residues, eight belong to structural residues (four cysteines and four glycines; Fig. 1a). Remarkably, except the lacking of two cysteines involved in the formation of the third disulfide bridge, AdDLP well matches the consensus motif of the eukaryotic defensins with a typical cystine-stabilized α-helix (CSH) sequence motif, a nested unit of the CSαβ motif (Tamaoki et al. 1998), conserved in position with Rorsins. To further confirm our observations, we scanned for the antimicrobial peptide (AMP) database using BLAST algorithm and *E*-value threshold of 1e−2, which again identified two types of defensins respectively from clusters 98 and 143 (Fjell et al. 2007) as the best match to AdDLP. This is further strengthened by fold recognition

using mGenTHREADER (Bryson et al. 2005), which assigns many known CSαβ peptides including some defensins as the best hits of AdDLP (Fig. S1a, as supplementary information of *Immunogenetics*). These features suggest that AdDLP could adopt a defensin-like fold with putative antimicrobial activity.

To test this hypothesis, we predicted the tertiary structure of AdDLP by comparative modeling (Fig. 2a). Comparative modeling is a computation-based structural biology approach that has successfully been used in the discovery of new insulin-like proteins from *C. elegans* (Duret et al. 1998) and scorpion neurotoxins from *M. martensii* (Zhu and Gao 2006). The model of AdDLP do favor the existence of the CSH structural motif comprising one α-helix (residues PQCKAYC) linking to the C-terminal β-strand (residues CVC) by two conserved disulfide bridges. Another β-strand is composed of residues GAI. In addition to conservation in the secondary structure elements, the AdDLP structure is globally very similar to that of plectasin with a RMSD of 0.78 Å for 37 Cα atoms (Fig. 2b). Two equivalent positions to the 4Cys and 30Cys of plectasin that are involved in the formation of the third disulfide bridge are occupied by 4Ser and 29Ile and are close each other in the structure of AdDLP. When 4Ser and 29Ile of AdDLP are substituted for Cys residues (named AdDLP3SS), a disulfide bridge with an optimal distance between two Cα atoms (6.16 Å) can be generated in a good structure model identified by Verify3D scoring above 0.2 (0.258; Fig. S2, as supplementary information of *Immunogenetics*). These observations provide a structural basis for emergence of the first CSαβ defensin in eukaryotic life by establishing this additional disulfide bridge on the bacterial DLP scaffold carrying the CSH structural motif. Of particular further analysis of the structure of AdDLP allowed us to identify a putative antimicrobial architecture of amphipathicity in which hydrophobic and hydrophilic structural clusters are spatially separated (Brogden 2005; Fig. 2c). Such a unique structural arrangement was also observed in the structure of plectasin (data not shown).

Evolution of two cysteines from structurally adjacent residue pair Ser and Ile to assemble a disulfide bridge has been observed in several CSαβ peptides. For example, a recent study performed by Zhu et al. identified a scorpion Na-channel toxin ancestor with only three disulfide bridges (Zhu and Gao 2006). At this ancestral scaffold, two point mutations (Ser/Cys and Ile/Cys) built the fourth disulfide bridge and finally led to the generation of diverse pharmacological groups. Another example can also be found in some members of the family III of fungal defensins, which also used this mechanism to evolve another disulfide bridge (Zhu 2008). Hence, it appears that

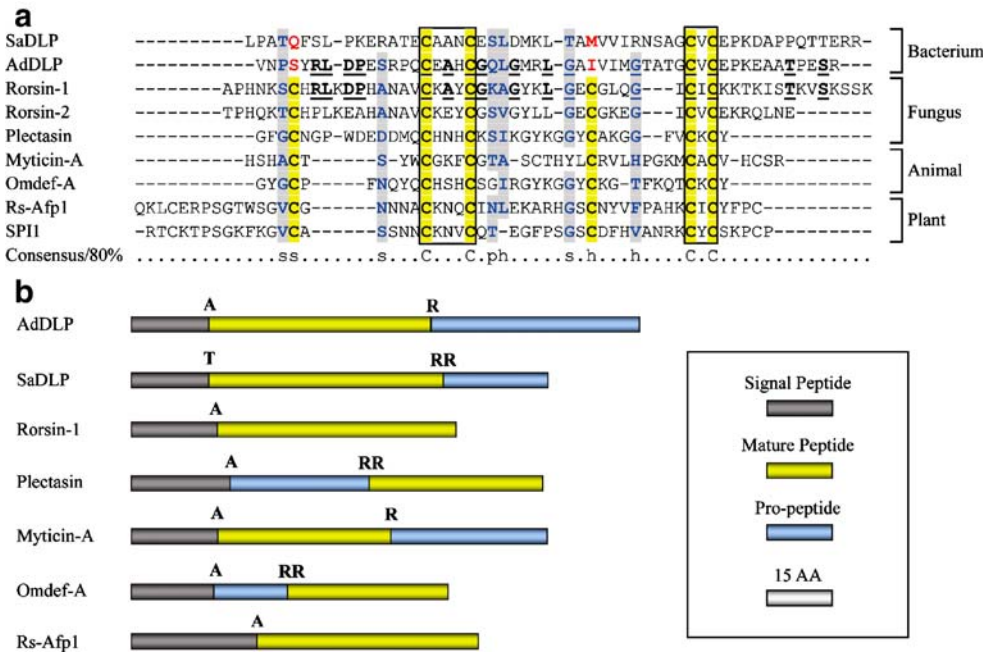

Fig. 1 Comparison of prokaryotic and eukaryotic defensins. **a** Sequence alignment of representative defensins. Data resource: AdDLP (YP_467026), SaDLP (ZP_01466688), Rorsin-1 and Rorsin-2 (Zhu 2008), Plectasin (Mygind et al. 2005), Myticin-A (Mitta et al. 1999), Omdef-A (Nakajima et al. 2001), Rs-Afp1 (Terras et al. 1995), and SPI1 (Sharma and Lönneborg 1996). Sequences involved in the formation of the CSH motif are *boxed*. *s* Small residue, *h* hydrophobic residue, *p* polar residue. Cysteines are *shadowed in yellow*, and identical residues between AdDLP and Rorsin-1 are *underlined once*. Residues in the bacterial DLPs that might develop into cysteines when evolving to eukaryotes are in *red*. **b** Precursor organization of representative defensins. Residues at the processing sites are shown here

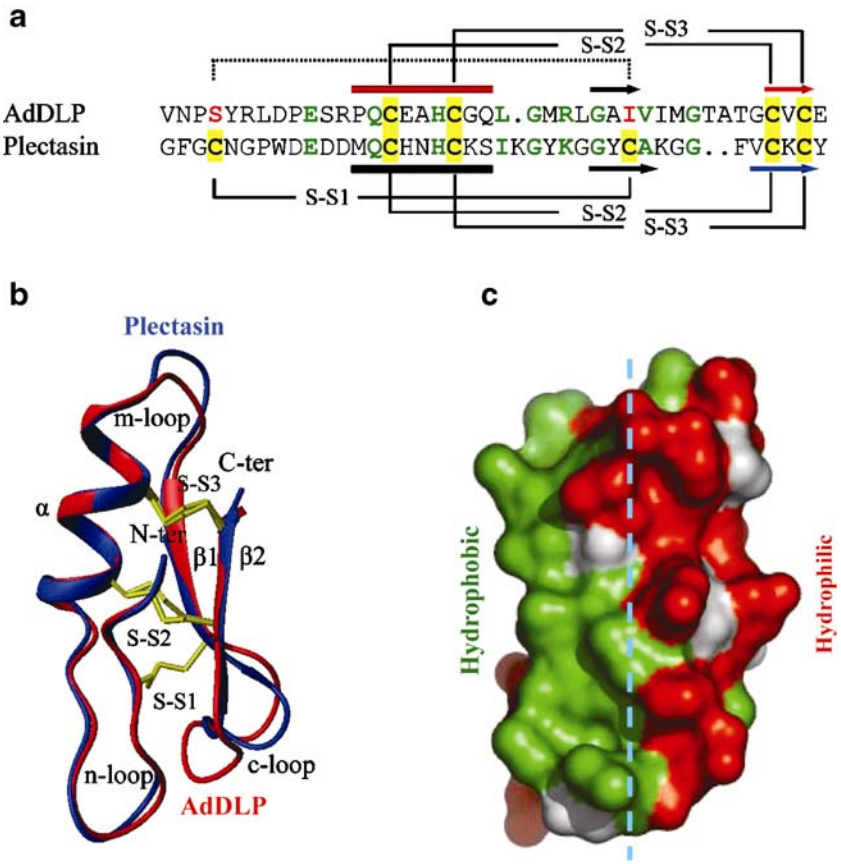

Fig. 2 Structural similarity between bacterial DLP and eukaryotic defensins. **a** Sequence alignment of AdDLP and plectasin for structural modeling. Identical residues and conserved replacement are shadowed in *green*. Disulfide pattern and secondary structure region are calculated from their structural coordinates. Lacking of the first disulfide bridge in AdDLP is indicated by *dotted lines*. Cylinders represent α-helices, and *arrows* represent β-strands. **b** Superimposition of AdDLP and plectasin structures. **c** Structural evidence for antimicrobial activity of AdDLP

the residue pair Ser and Ile could represent a hot spot for the development of new disulfide bridge.

Although low sequence similarity existing between SaDLP and eukaryotic DLPs, clear orthologous relationship between SaDLP and AdDLP and sharing of a conserved CSH sequence motif suggest that SaDLP might also adopt a defensin-like fold, as revealed by fold recognition (Fig. S1b, as supplementary information of *Immunogenetics*).

Discussion

Bacteria constitute a major pathogen of early eukaryotic life when it emerged from prokaryotes by the possible endosymbiosis mechanism (López-García and Moreira 2006). However, how these early eukaryotic organisms can resist prokaryotic microbial infection is largely unknown at present. According to *the Syntrophic Hypothesis* proposed by Moreira and López-García (1998), it is very likely that an ancestral myxobacterium could contribute its defense system to early eukaryotes by horizontal gene transfer of its immune-related genes to the proto-eukaryotic genome during eukaryogenesis. The identification of two defensin-like peptides in the myxobacteria and the confirmation of their restricted distribution to myxobacteria have important implications for elucidating prokaryotic origin of innate immunity effectors of eukaryotic life. Evidences supporting the hypothesis that the eukaryotic defensins originated from one ancestral myxobacterial DLP come from the following observations:

1. At the structural level: assembly of additional disulfide bridges in an ancestral scaffold represents a common strategy for proteins to evolve new structure and function. Such a strategy has been proposed to: (1) explain the relationship of the ancient disulfide-directed β-hairpin fold and inhibitor cystine knot fold (Wang et al. 2000), (2) elucidate the origin of scorpion sodium channel neurotoxins (Zhu and Gao 2006) and type 2 cystatins (Müller-Esterl et al. 1985), and (3) represent a coevolutionary mechanism to direct and shape the

diverse repertoire of AMPs to overcome microbial resistance (Peschel and Sahl 2006). This is also consistent with the prevailing view that disulfide bridges have been added during evolution to enhance the stability of proteins that functions in a fluctuating cellular environment (Hogg 2003).

From a structural viewpoint, the CSαβ motif is actually a minor elaboration of a simpler ancestral motif, known as the CSH motif that consists of a pair of cysteines located on the α-helix and separated by three amino acids (CXXXC). This pair of cysteines is connected via two disulfides to a second pair of cysteines (CXC), itself folded in an extended β-strand-type structure (Tamaoki et al. 1998). Peptides with the minimum CSH motif might hold an ancestral role for the evolution of the CSαβ motif. However, the CSH peptides previously characterized such as the members of the ET/SRTX family or the bee venom toxins only represent a consequence of structural convergence other than evolutionary relationship because of the inverse orientation in the CSH motif (Tamaoki et al. 1998). On the contrary, AdDLP and SaDLP may represent the real archetype of the CSαβ-type defensins owing to identical orientation in their CSH motif, which can easily serve as a platform to assembly different types of defensins by evolving disulfides in different positions. Therefore, it appears that all the CSαβ defensins have evolved from a single common ancestor, being a molecule with a primordial CSH motif and only two disulfides structurally resembling the bacterial DLPs.

2. From the viewpoint of the origin of eukaryotes: as predators of other microorganisms (Reichenbach 1999), the myxobacteria have been considered as a bacterial partner involved in the origin of eukaryotic life by endosymbiosis of a methanogenic archaeon (Moreira and López-García 1998; López-García and Moreira 2006). The latter formed early eukaryotic nuclear genome (proto-eukaryotic genome) and obtained some genes of the myxobacteria by horizontal gene transfer in the subsequent evolution. Considering eukaryotic defensins having a limited antibacterial spectrum and being highly active against many Gram-positive bacteria but generally not being able to inhibit the growth of majority of Gram-negative bacteria (Dimarcq et al. 1998; Lay and Anderson 2005), it is reasonable to assume that the Gram-negative myxobacteria are likely resistant to their own DLPs, although the functional data of these two bacterial DLPs is not available at present. The existence of the DLP in the myxobacteria may thus be helpful to defend some Gram-positive microbial infection in their common habitat, as do some antibiotics. If our inference is correct, it is possible that the myxobacterial DLPs, as early transferred immune-related genes, protected ancestral eukaryotes from bacterial infection. To confirm this inference, it is needed to characterize the bacterial targets of these two DLPs. Given most of antibacterial peptides from bacteria have narrow inhibitory spectra (Diep and Nes 2002), it could be a fascinating problem about how one can identify a microbial target for such highly selective antimicrobial molecules. At present, such investigation is under progress. However, regardless of the antibacterial activity of these two peptides, their structural feature provides evidence for a key role in the origin of eukaryotic defensins.

It is also worth mentioning that 15 deltaproteobacterial species whose genome sequences have been completed (http://www.ncbi.nlm.nih.gov/) lack orthologs of AdDLP/SaDLP. This seems to suggest that such peptides could be the product of horizontal gene transfer from an unidentified eukaryotic genome. However, gene loss after speciation probably is a more plausible explanation, which are supported by the following facts: (1) the lack of AdDLP gene in the genome of *Anaeromyxobacter* sp. Fw109-5, a closely related sibling species of the *A. dehalogenans*, clearly shows that gene loss event occurred during evolution, (2) the existence of DLPs in two distant bacterial genomes (*A. dehalogenans* and *S. aurantiaca*) supports common ancestry, (3) Adding of disulfide bridges in an ancient scaffold represents an evolutionary advantage in stabilizing protein structure (Hogg 2003). Removal of the third disulfide from a eukaryotic DLP presumably transferred from the myxobacteria appears unlikely.

Previously, Boman's group has proposed that the insect antibacterial peptides—cecropins have evolved from ribosomal protein L1 of an ancestral intracellular pathogenic bacterium (Putsep et al. 1999a, b). Although differing in the bacterial partnership, both cecropins and defensins, two most famous insect antibacterial peptides, appear to originate considerably early, which can be traced to prokaryotic life time when these ancestral molecules could perform a similar task to the present-day antimicrobial peptides.

In conclusion, successful identification of two bacterial peptides with a conserved CSH motif gives us an opportunity to elucidate the evolutionary history of the eukaryotic defensins. With them in hand, now we can design experiments to determine their structure and function as antibacterial factors in the immune response of the myxobacteria from ecological and evolutionary perspective. Furthermore, adding of the additional disulfide bridge corresponding to that of the eukaryotic defensins on the scaffold of AdDLP and SaDLP will help us to evolutionarily mimic the origin process of the eukaryotic defensins in laboratory.

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