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Research Article

Evolutionary and functional epitopes of the Spätzle protein: New insights into activation of the Toll receptor

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Abstract. Spätzle, a dimeric ligand, binds to the *Drosophila* Toll receptor and activates the signal pathway functioning in both embryonic patterning and innate immunity. Here, we used the evolutionary trace approach based on phylogenetic information to predict the evolutionary epitope of Spätzle and found that it mainly clusters in several adjacent loops of Spätzle far from the cystine-knot structural domain. We designed six mutants of Spätzle based on the evolutionary epitope and transfected them into a stable cell line expressing the luciferase reporter gene under the control of the *drosomycin* promoter. Luciferase assays showed that these mutants cannot significantly activate the *drosomycin* promoter, suggesting the involvement of these sites in binding of Spätzle to the Toll receptor. These data highlight the importance of the Trp-loop of the mushroom-shaped Spätzle dimer in Toll receptor activation and demonstrate that evolution-guided site-specific mutagenesis represents a useful and promising strategy for understanding the ligand-receptor interaction.

Keywords. Innate immunity, Toll pathway, *Drosophila*, evolutionary tracing, protein-protein interaction.

Introduction

The Toll signal pathway plays key roles in regulating the innate immunity response of invertebrates and vertebrates. In *Drosophila*, besides acting in embryonic development, the Toll pathway is essential for the regulation of inducible expression of a subset of antimicrobial peptides (AMPs) against fungal and Gram-positive bacterial infection [1, 2]. Spätzle, as an extracellular ligand for the Toll receptor, is required for activation and signaling of the Toll pathway [3–6]. The dimeric precursor of Spätzle is processed by endoprotease to produce an active form comprising 106 amino acids of C-terminus (called C106) that cross-links the Toll receptor ectodomain and establishes signaling [3–10]. The structure of C106 forms a parallel dimer covalently linked by an intermolecular disulfide bond between CysA98 and CysB98 [1, 9, 11]. Spätzle (Spz1) has five *Drosophila* homologues (Spz2–6) which encode proteins containing the neurotrophin-like cystine knot (CK) structural motif and most of them retain a characteristic intron-exon structure [11], suggesting that this family arose by gene duplication and these Spätzle homologues could function as activating ligands for corresponding Toll receptors [11].

Although the three-dimensional (3D) structure and biological roles of Spätzle have been well documented, its functional surfaces involved in activation of the

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Toll receptor are still unknown. Early studies suggested that C106 specifically binds to Toll with a stoichiometry of one Spätzle dimer to two receptors [1, 12]. However, this model has recently been modified by Gangloff *et al.*, who proposed that the Spätzle dimer bound to the N-terminal end of Toll can predominantly induce the formation of a 2 : 2 complex [13]. Thanks to the determination of the crystal structure of C106 [14], it now becomes possible to structurally map the functional residues of this ligand and this will undoubtedly be useful for further studying a detailed interaction mode between Spätzle and Toll in a structural context.

In this paper, we applied the evolutionary trace (ET), a computational method of genetics analysis [15–18], to predict evolutionarily privileged sites (evolutionary epitope) of Spätzle and used them to direct site-directed mutagenesis for elucidation of the functional surface of Spätzle. The results presented here for the first time highlight a key role of the head (the Trp-loop) of the mushroom-shaped Spätzle dimer in binding the Toll receptor.

Materials and methods

Database searches of Spätzle homologues. *Drosophila melanogaster* Spätzle protein sequences including Spz1–6 retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) (CG6134, CG18318, CG7104, CG14928, CG9972 and CG9196) were used as queries to search for new homologues. An initial PSI-BLAST (Position-Specific Iterated BLAST) search was used to collect homologues of Spätzle containing a CK domain [11] in the none-redundant (nr) database. PSI-BLAST converged after five rounds of interactions using the E-value cut off of 0.005. We obtained the signature pattern of Spätzle from the last round collection that can be described as $CX_8P\ldots\ldots QX_6C\ldots\ldots C\ldots\ldots C\ldots\ldots PXXCXC$, where X is any amino acid. Then we performed TBLASTN using the amino acid sequences collected as queries to search the Genbank, VectorBase (http://www.vectorbase.org/index.php), Gene Index Databases (http://compbio.dfci.harvard.edu/index.html), Human Genome Sequencing Center at Baylor College of Medicine (HGSC) (http://www.hgsc.bcm.tmc.edu/projects/) and Silkworm Genome Project (http://silkworm.genomics.org.cn/jsp/data.jsp, http://sgp.dna.affrc.go.jp/, http://silkworm.swu.edu.cn/blast/blast.html). All hits with an E-value of less than 10 were examined manually for the conserved signature obtained from PSI-BLAST, especially the residues involved in the maintenance of neurotrophin-like CK fold [11]. All sequences judged by these criteria as possible Spätzle homologues were again used as new queries for next rounds of TBLASTN. Searches repeated until no new hits appeared. Homologues identified here were named according to the known *Drosophila* Spätzles. The intron-exon structure of a Spätzle gene was determined by MGAlignIt (http://origin.bic.nus.edu.sg/mgalign/) and Wise2 (http://www.ebi.ac.uk/Wise2/).

Evolutionary trace analysis. A total of 68 Spätzle and Spätzle-like proteins were obtained from 20 model insect species (*Culex pipiens*, *Aedes aegypti*, *Anopheles gambiae*, *Bombyx mori*, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Pediculus humanus corporis* and 12 *Drosophila* species). To eliminate the effect of potentially misleading sequence alignment resulted from the existence of some large gaps, 66 were retained and used for the evolutionary trace analysis (for sequence information, see Figure S1 and Table S1, provided as Supplemental data). Multiple sequence alignment (MSA) was performed using the program CLUSTALX [19] and then refined manually. The ET analysis was carried out on the basis of the phylogenetic tree created by the unweighted pair group method with arithmetic mean [20]. Here, the ET method divided all residues of aligned sequences into three classes: neutral, conserved and class-specific, based on comparing the consensus sequences for groups of proteins which originate from a common node defined by the evolutionary time cut-off (ETC) in a phylogenetic tree [15–18]. The smallest number of branches at which one position becomes invariant within each branch defines its rank. Gaps are counted as an extra residue type and confer neutrality on the trace at positions where they occur in the MSA [15]. The class-specific residues are the most interesting in terms of the development of functional innovation during evolution. The evolutionary epitope of Spätzle is structurally mapped by PyMOL (http://pymol.sourceforge.net). Considering the long loops from residues G^{22} to N^{35} of chain A and from residues L^{23} to D^{36} of chain B are disordered in the crystal structure of Spätzle (PDB code 3E07) [14], we used the energy minimum approach to model these loops at the server (http://bioserv.cbs.cnrs.fr/HTML_BIO/ frame_home.html) and used this modified structure to map the evolutionary epitope. The 3D protein model of this modified structure has been submitted to the Protein Model database (http://www.caspur.it/PMDB/) under the id number of PM0075630.

Site-directed mutagenesis of the evolutionary epitope. Primers for constructing mutants and DNA sequencing are provided in Table 1. The Spätzle expression plasmid (pJM856) [21] was constructed by fusing signal peptide

Table 1. Primers used in this study

Mutants	Name	Sequence
Y18A	18AF	5'-GCc cca aaa aag ggc ttg agg gcg gac g-3'
	18AR	5'-cac cag ctt cct gat gct cct gca aag-3'
P19A	19AF	5'-tac Gca aaa aag ggc ttg agg gcg gac g -3'
	18AR	5'-cac cag ctt cct gat gct cct gca aag-3'
I32A	32AF	5'-GCt gtc aat aac gat gag tac aaa cag-3'
	36AR	5'-taa ctg cca ggt gtc gtc cgc cct ca-3'
N34A	34AF	5'-att gtc GCt aac gat gag tac aaa cag-3'
	36AR	5'-taa ctg cca ggt gtc gtc cgc cct ca-3'
$I^{32}VNN^{35}$ deletion	36AF	5'-gat gag tac aaa cag gcc atc cag atc g-3'
	36AR	5'-taa ctg cca ggt gtc gtc cgc cct ca-3'
D87A	87AF	5'-gCc gtg gtg cag aat tcc ttc aag at-3'
	87AR	5'-cag ctc gcc atc act ctt gat gct g-3'
—	Spz-seq	5'-atgacgcccatgtggata-3'

Note. Mutated bases are indicated in uppercase. All primers listed here were synthesized in SBS Genetech (Beijing, China).

directly to the C106 amino acids. Inverse PCR, as previously described [22], was used to generate all six mutants (Y18A, P19A, I32A, N34A, D87A and $I^{32}VNN^{35}$ deletion) by using the plasmid pJM856 as a template. Phosphorylation of the 5'-end of primers was performed using polynucleotide kinase (TOYOBO, Osaka, Japan) and ATP (Takara, Dalian, China). Reaction conditions were as follows: 18 cycles of 45 sec at 94 °C, 30 sec at 55 °C, and 10 min at 72 °C with Ex Taq polymerase (Takara, Dalian, China). Subsequently, linear PCR products were circularized by T4 DNA ligase (Takara, Dalian, China) after end polishing using Pfu polymerase (newProbe, Beijing, China). Circularized products were transformed into *E. coli* DH5α competent cells. Positive clones were confirmed by DNA sequencing using the primer Spz-seq (Table 1) and their plasmids were extracted for transfection.

Luciferase assays. The stably transfected *Drosophila* S2 cell line (648–1B6) expressing the luciferase reporter gene under the control of the *drosomycin* promoter [1] was used in this work. Cells were grown at 25 °C in Schneider's medium (Sigma, St Louis Missouri, US) supplemented with 10% fetal calf serum, 10^5 units/liter penicillin, 100 mg/liter streptomycin, and 1μg/ml puromycin. Cells were transfected in 6 cm diameter dishes by the calcium phosphate precipitation technique with 0.1 μg of β-galactosidase expression vector pACH110 and 1μg of expression vector [21]. After 48 h, cells were lysed in reporter lysis buffer and luciferase activity was measured in Modulus™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) immediately after adding of the substrate luciferin (Promega, Madison, WI, UA). β-galactosidase activity in the cell lysates was measured by using O-nitrophenyl-β-D-galactoside as the substrate (Promega, Madison, WI, UA), and values were used to normalize variability in the efficiency of transfection [21].

Results

Phylogeny of the Spätzle family. Taking advantage of the signature built from PSI-BLAST and TBLASTN search, we identified a total of 68 Spätzle and Spätzle-like proteins distributed in 20 model insect species, in which 66 were used here for ET analysis. Because the CK domain of Spz6 is longer than that of Spz1–5, we removed the middle region of these proteins to obtain a reliable alignment. On the basis of this alignment, some structural features of this family can be drawn here. Firstly, the CK domain of Spätzle identified here is composed of about 106 residues with some residues conserved across the entire alignment, which include six cysteines, two prolines, and one glutamine (Figure S1). The six conserved cysteines are crucial to disulfide formation and fold stability of the CK motif, in which the ring formed by two disulfide bonds (Cys2-Cys5, Cys3-Cys6) and their connecting backbone segments is penetrated by the third disulfide bond (Cys1-Cys4). Secondly, most of Spätzle members have, in front of Cys5, an additional cysteine to facilitate dimerization by forming a covalent disulfide bridge between the two monomers [6, 14, 23]. Spz3 and Spz4 have two additional cysteines located between Cys4 and Cys5. Thirdly, most of variable regions in length were found to be located in some loops between anti-parallel β-strands in Spz1–6, which are named L1, L2, L3, L4 and L5 (Figure S1).

When 66 Spätzle sequences were taken into consideration for phylogenetic analysis, the tree split these sequences into six distinct subfamilies (Spz1–6) (Figure 1), consistent with the previously described

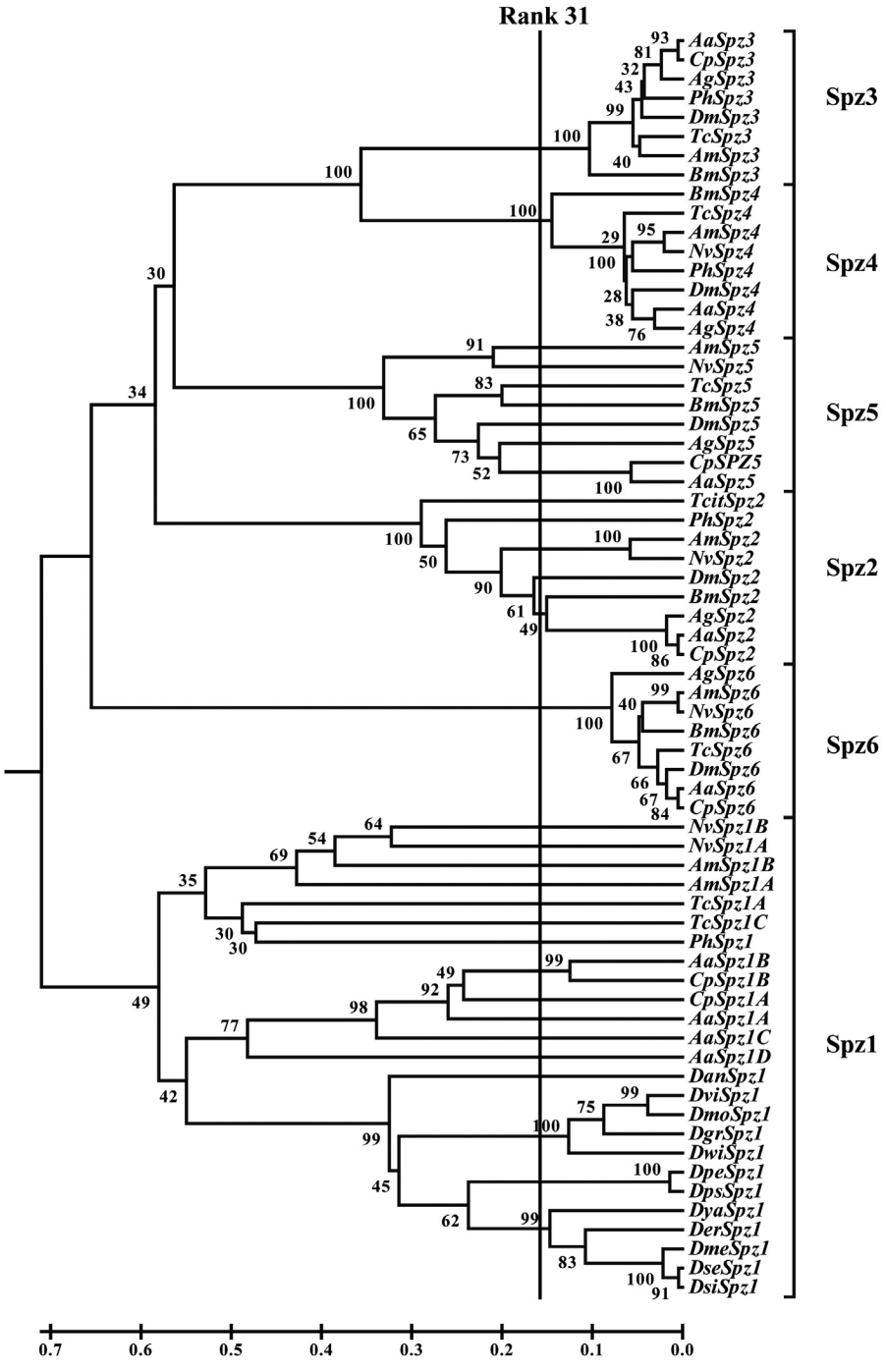

Figure 1. The UPGMA phylogenetic tree of the Spätzle family. This tree was constructed from amino acid sequences of C106. The root was placed at the midpoint of the longest span across the tree. A vertical line divided the tree into the specified number of branches (called rank) and indicated functional resolution of ET at this point. The class-specific residues were filtered by rank 31. Six distinct subgroups (Spz1–6) were identified by the tree.

nomenclature [11]. The relationship within each subfamily of Spätzle agrees roughly with their species phylogeny, suggesting gene duplication of *Spätzle* and subsequent evolution occurred before species divergence. For example, the Spz1 of dipteran, including *Drosophila*, *Aedes*, *Culex*, *Anopheles*, always clustered together. In Spz1, 2, 4, 5 and 6, holometabolous insects such as *Apis* and *Nasonia* are also in the same clade. Proteins within each subfamily from Spz2–6 show high sequence conservation (70 % –95 %). But peptides from different subfamilies are more divergent among each other. Spz1 represents the most diverse subfamily with only about 40 % sequence identity.

Evolutionary epitopes of the Spätzle family. ET extracted 14 evolutionarily privileged sites (Figure 2A) at rank 31. Three residues are conserved across the Spätzle family, including two hydrophobic residues (P^{19} and P^{96}) and one hydrophilic residue (Q^{40}), as

previously mentioned. Other 11 ET residues such as L^{9}, G^{22}, N^{34}, Q^{43}, E^{45}, Q^{75}, L^{77}, A^{78}, D^{87}, V^{88} and F^{93} were identified as class-specific, among which N^{34} and E^{45} are conserved across subfamilies Spz1 to Spz5 (residues are numbered according to *D. melanogaster* Spätzle C106). To obtain a complete Spätzle structure to map these ET residues, we built the structures of the long loops from residues G^{22} to N^{35} of chain A and from residues L^{23} to D^{36} of chain B disordered in the crystal structure of Spätzle through the energy minimum approach and found the whole dimer presents a mushroom shape (Figure 2B), in which L2 (i.e. the Trp-loop) is the biggest loop (Figure 2A and 2C). When locating ET residues on the Spätzle structure, we found that most of them cluster in several adjacent loops (Figure 2C). These loops are mostly centered on the head of the mushroom-shaped structure (Figure 2B). A detailed analysis of the ET result indicates most of the residues likely play key structural roles based on the following reasons: *1*) As highlighted in the 3D structure, residues L^{9}, Q^{40}, Q^{43} and E^{45} are almost located on the interface of the Spätzle dimer with less accessibility, in which E^{45} is in contact with R^{14}, K^{100} and H^{71} (K^{100} and H^{71} coming from another monomer) and Q^{43} contacts R^{14} [14]. Moreover, L^{9}, Q^{43} and E^{45} are close to the CK domain of the dimer axis. Q^{40}, a key structural residue conserved among coagulogen, nerve growth factors (NGFs) and NGF-related sequences, could also have a similar role in Spätzle [11, 23]; *2*) Structure-based sequence alignments of NGF, coagulogen and Spätzle identified some similar residues which comprise a hydrophobic core in the NGF structure including V^{36}, V^{38}, F^{53}, A^{89}, I^{102} and I^{104} (residues are numbered according to the crystal structure of NGF, PDB code 1BET) [23]. Such hydrophobicity is also conserved in the Spätzle except for the first residue, and the residues in the NGF corresponding to I^{32}, I^{44}, L^{77}, F^{93} and I^{95} of Spätzle. F^{93} is located on the interface of the dimer. Moreover, residues N^{91}-F^{93} may act as a hinge linking the disulfide-linked core and the 'wings' [14]. *3*) ET residues Q^{75}, L^{77}, A^{78} and P^{96} are mostly situated on β-strands and can also be assumed to be important in structural stability.

However, two ET residues (P^{19} and N^{34}), highly exposed on the molecular surface with $> 30\,\%$ solvent accessibility, are ideal target sites selected for mutation. In addition, D^{87}, a negatively charged residue on the small β-strand, was also selected in order to observe a possible charge effect in the ligand-receptor interaction. For comparison, we also mutated two additional sites (Y18A and I32A) adjacent to the two ET residues exposed on the Trp-loop (I^{32} close to N^{34} and Y^{18} close to P^{19}). To further evaluate the importance of this loop, we also designed a mutant with $I^{32}VNN^{35}$ deleted.

Functional consequence of Spätzle mutants. *In vivo* genetic evidence in *Drosophila* larvae and adults indicates that a processed form of the secreted protein Spätzle can efficiently activate the Toll signal pathway [3–6] and induce the expression of *drosomycin* [1, 24], whereas *in vitro* results showed that the processed form of Spätzle markedly activated the *drosomycin* promoter in the *Drosophila* S2 cells which were cotransfected with a vector expressing the Spätzle C106 and a reporter construct expressing luciferase under the control of the *drosomycin* promoter [21]. Considering that *in vitro* accurate quantification between control and test groups is easier to fulfill than *in vivo*, we therefore chose the former system to evaluate of the functional consequence of six Spätzle mutants. The derivative (648–1B6) of the macrophage-like *Drosophila* S2 cell line carrying a reporter construct expressing luciferase under the regulation of the *drosomycin* promoter [1] was used to compare the activating effect of the *drosomycin* promoter under different *Spätzle* mutants. Cells were cotransfected with a *Spätzle* or its mutant expressing vector together with the β-galactosidase expression vector. Luciferase assays illustrated that the *drosomycin* promoter was dramatically activated four-fold by the processed form of Spätzle (Fig. 3), consistent with the reported result [21]. As for the mutants, there was a significant discrepancy between the control (the wild-type Spätzle) and the test (Y18A, P19A, I32A, N34N, $I^{32}NVV^{35}$ deletion, D87A). Under the regulation of Spätzle mutants, the luciferase level (= activation of the *drosomycin* promoter) is statistically lower than the wild-type Spätzle ($p<0.05$) (Fig. 3), suggesting that all six Spätzle mutants showed lower efficiency in triggering the activation of the *drosomycin* promoter. Moreover, mutations of I32A and $I^{32}NVV^{35}$ deletion, which are all located on the Trp-loop, significantly decreased the activation of the *drosomycin* promoter ($< 25\,\%$) relative to the wild-type Spätzle, and were also lower than other mutants ($p < 0.05$) except for Y18A (Fig. 3). In fact, the mutation in I32A or deletion in $I^{32}VNN^{35}$ almost completely abolished the activating potency of Spätzle when compared with the empty vector. These results indicated that these sites are likely involved in binding of Spätzle to Toll, in which a key role for the Trp loop is clearly highlighted.

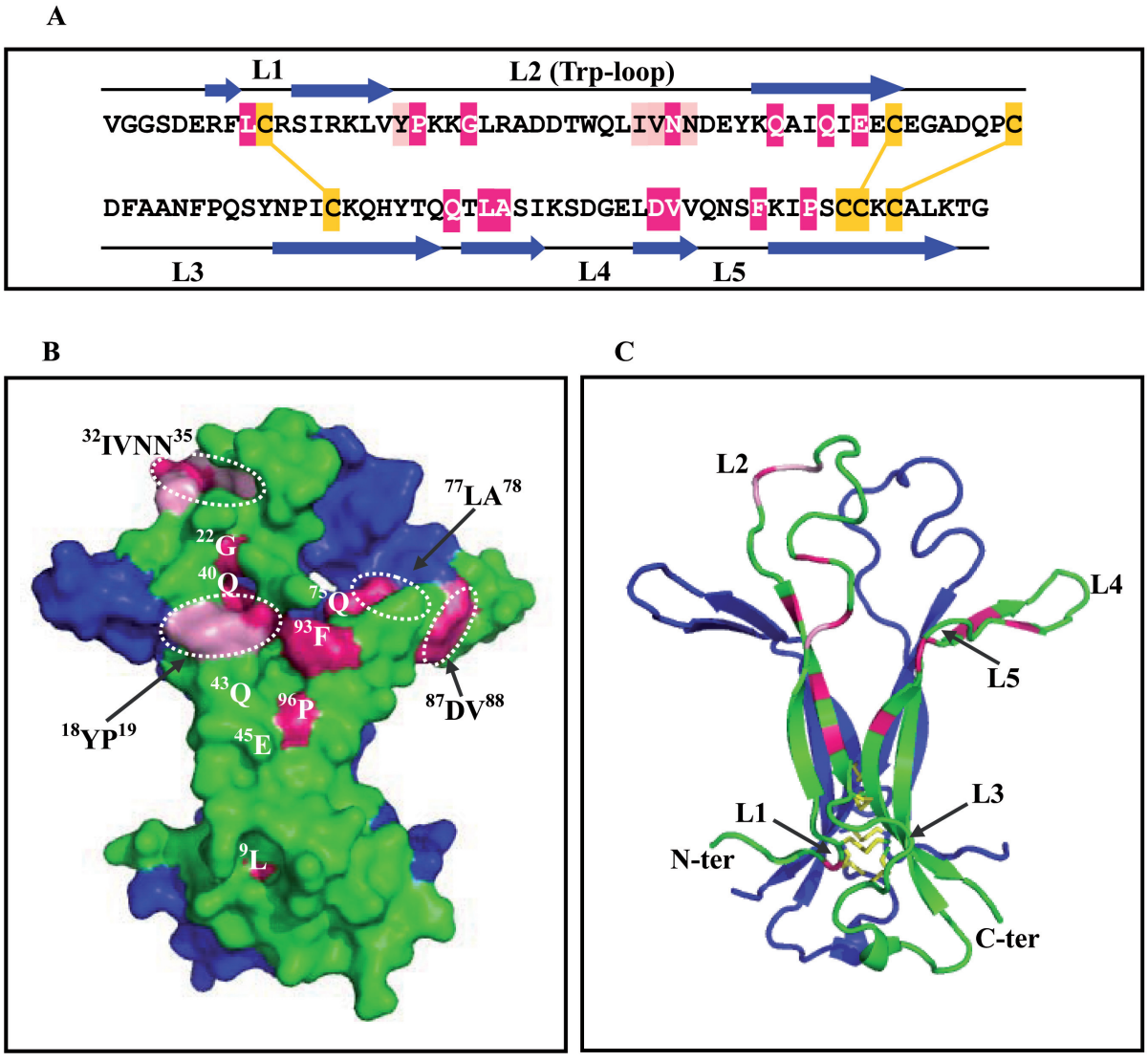

Figure 2. The evolutionary epitope of Spätzle. *A.* Amino acid sequence and secondary structural element of Spätzle. Trace residues are shadowed in hot pink, cysteines in yellow and four residues (Y^{18}, I^{32}, V^{33} and N^{35}) adjacent to the evolutionary epitope in pink; *B.* The molecular surface of the C106 dimer showing the location of the evolutionary epitope; C. Secondary structure cartoon of Spätzle with five loops indicated. Color codes used in Figure 2B and 2C are the same as those in Figure 2A.

Discussion

Spätzle is the only known ligand of the Toll receptor which activates the pathway by its C-terminal mature segment (C106) binding to the N- terminus of Toll [3]. *In vitro* experiments show that Spätzle binds to the Toll ectodomain with high affinity and with a stoichiometry of one Spätzle dimer to two receptors [1]. However, the electron microscopy structures of the Toll ectodomain in the absence and presence of Spätzle indicates that the Spätzle dimer does not directly cross-link two Toll ectodomains, instead binding at the N-terminal end of Toll [13]. Such binding predominantly induces the formation of a 2 : 2 complex. Also, Toll undergoes a ligand-induced conformational change and becomes more tightly curved than in the apo form. Mass spectrometry studies [13] revealed that the 2 : 2 complex was predominant over the 2 : 1 described earlier [12]. Finally, the electron microscopy structure of the complex suggests that activation of Toll is an allosteric mechanism induced by an end-on binding mode of its ligand. Therefore, a logical next step is to know the orientation of the elongated Spätzle dimer binding the Toll solenoid in an end-on configuration. Elucidating the functional surface and determining the binding end of Spätzle are major purposes of this study.

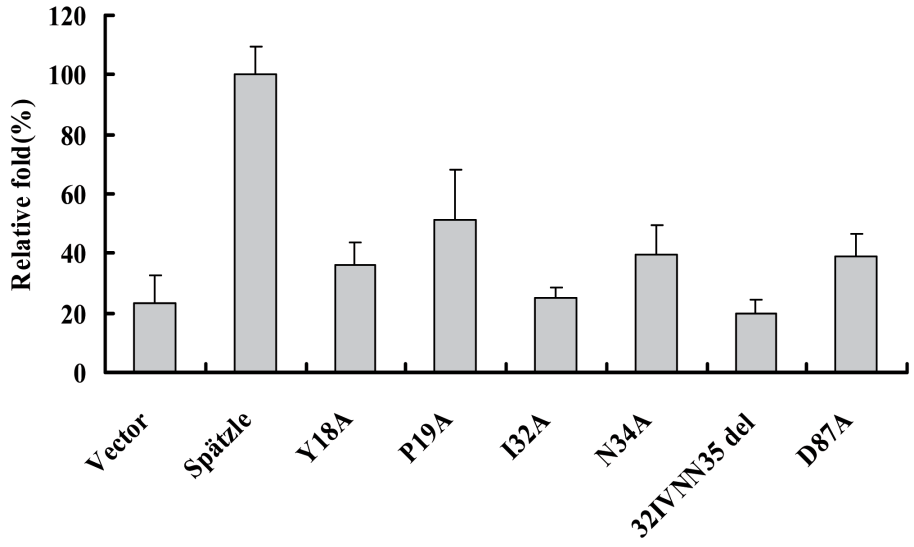

Figure 3. *Drosomycin* promoter luciferase reporter assay in *Drosophila* S2 cells. 648–1B6 cells were cotransfected with the empty vector or a recombinant vector carrying C106 or a mutant together with β-galactosidase. All transfections were done in triplicate, and results represent means ± SD.

Clearly, traditional analysis of a protein functional surface requires undergoing a systematic and exhaustive mutation [25]. In this case, numerous technical difficulties associated with their expression, purification and structural characterization present a big challenge to researchers. Phylogenetic relationships could provide valuable information about the functional significance of a particular residue or region of a protein. ET analysis is a method developed to utilize this phylogenetic information to predict functional sites in proteins [15, 16] and has been successfully used in different biological systems [17, 26–35]. For example, Zhu *et al.* successfully highlighted one channel-binding surface common for the scorpion α-KTx family of neurotoxins [34]. Supported by these facts, we believe that it is also possible to use evolution-guided information to identify key residues of Spätzle and then to direct further mutational analyses.

In this study, we selected three trace residues for substitution by alanine, in which one was also chosen for deletion along with its three neighboring amino acids. In addition, given neighboring amino acids around trace residues could also impact the protein function (personal communication with Prof. Olivier Lichtarge), we mutated two such residues with solvent accessibility >30%. Our mutation data for the first time highlighted the Trp-loop as a key functional determinant for Spätzle binding to Toll (Fig. 4), consistent with previous structural studies [14]. However, it is worth mentioning that other minor determinants of activation may exist, because some mutations can also affect the binding of Spätzle to Toll.

In conclusion, our studies provide new clues for a deeper understanding of the activation mode of the Toll signal pathway and are also likely useful in designing new small molecules based on the Trp-loop to target the pathway and regulate the immune response of *Drosophila*. Despite the fact that not all trace residues are equally crucial for the binding, mutations of trace residues nonetheless represent a rational design for studying interaction between Spätzle and Toll.

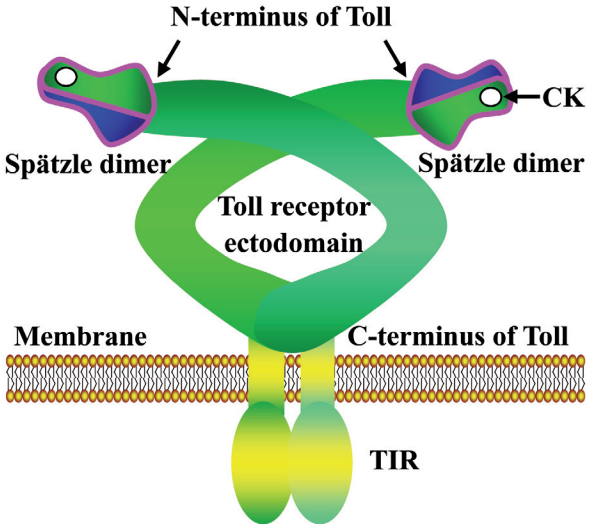

Figure 4. Schematic diagram of binding mechanism between Spätzle and Toll. This model is from Gangloff et al. [13], with an improvement based on our mutational data, in which the functional importance of the Trp-loop in the head of mushroom-shaped structure is highlighted.

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-009-9028-3) and is accessible for authorized users.

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