Research Article

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

Y. Wang and S. Zhu

Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (China), Fax: 0086 010 64807099, e-mail: Zhusy@ioz.ac.cn

Received 14 January 2009; received after revision 14 February 2009; accepted 09 March 2009
Online First 25 March 2009

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
Toll receptor are still unknown. Early studies suggested that C106 specifically binds to Toll with a stoichiometry of one Sp/C228tzle dimer to two receptors [1, 12]. However, this model has recently been modified by Gangloff et al., who proposed that the Sp/C228tzle 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 Sp/C228tzle 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 Sp/C228tzle and used them to direct site-directed mutagenesis for elucidation of the functional surface of Sp/C228tzle. The results presented here for the first time highlight a key role of the head (the Trp-loop) of the mushroom-shaped Sp/C228tzle dimer in binding the Toll receptor.

Materials and methods

Database searches of Sp/C228tzle homologues. Drosophila melanogaster Sp/C228tzle protein sequences including Spz1–6 retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/CGI-GEN Information Gateway) were used as queries to search for new homologues. An initial PSI-BLAST (Position-Specific Iterated BLAST) search was used to collect homologues of Sp/C228tzle containing a CK domain [11] in the non-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 Sp/C228tzle from the last round collection that can be described as CX, , 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 Sp/C228tzle 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 Sp/C228tzles. The intron-exon structure of a Sp/C228tzle gene was determined by MGAlignIt (http://origin.bic.nus.edu.sg/malign/) and Wise2 (http://www.ebi.ac.uk/Wise2/).

Evolutionary trace analysis. A total of 68 Sp/C228tzle and Sp/C228tzle-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 Sp/C228tzle is structurally mapped by PyMOL (http://pymol.sourceforge.net). Considering the long loops from residues G2 to N35 of chain A and from residues L2 to D36 of chain B are disordered in the crystal structure of Sp/C228tzle (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 Sp/C228tzle expression plasmid (pJM856) [21] was constructed by fusing signal peptide...