Toll-Like Receptors Are Part of the Innate Immune Defense System of Sponges (Demospongiae: Porifera)

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During evolution and with the emergence of multicellular animals, the need arose to ward off foreign organisms that threaten the integrity of the animal body. Among many different receptors that participate in the recognition of microbial invaders, toll-like receptors (TLRs) play an essential role in mediating the innate immune response. After binding distinct microbial components, TLRs activate intracellular signaling cascades that result in an induced expression of diverse antimicrobial molecules. Because sponges (phylum Porifera) are filter feeders, they are abundantly exposed to microorganisms that represent a potential threat. Here, we describe the identification, cloning, and deduced protein sequence from 3 major elements of the poriferan innate response (to bacterial lipopeptides): the TLR, the IL-1 receptor–associated kinase-4–like protein (IRAK-4l), and a novel effector caspase from the demosponge Suberites domuncula. Each molecule shares significant sequence similarity with its homologues in higher Metazoa. Sequence homologies were found in particular within the family-specific domains toll/interleukin-1 receptor/resistance (TLR family), Ser/Thr/Tyr kinase domain (IRAK family), and CASp (caspase family). In addition, in situ hybridization and immunohistological analyses revealed an abundance of SDTLR (TLR) transcripts in epithelial layers of the sponge surface (exopinacoderm and endopinacoderm). Furthermore, it is shown that both SDTLR and SDIRAK-4 like (IRAK) are expressed constitutively, regardless of treatment with synthetic triacyl lipopeptide Pam3Cys-Ser-(Lys)4. In contrast, SDCASL (caspase) expression is highly Pam3Cys-Ser-(Lys)4 inducible. However, blocking of the lipopeptide with recombinant TLR prior to its application completely prevented the induced expression of this poriferan caspase. These results underscore that the phylogenetically oldest extant metazoan phylum is provided already with the signaling pathways of the antimicrobial host-defense system of Metazoa.

Introduction

Innate immunity represents an efficient and complex first-line defense system that allows immediate response to microbial assaults. It is based on a set of germ cell–encoded receptors that are expressed at the surface of cells situated at host–environment boundaries. These evolutionarily conserved molecules were termed pattern recognition receptors (PRRs) because in their function as sentinels of the innate immune system, they recognize highly conserved microbial structures that were termed pathogen-associated molecular patterns (PAMPs). PAMPs are essential for survival and pathogenesis of microorganisms, often representing a molecular signature characteristic of the pathogen class (Medzhitov and Janeway 2000). The recognition of such structures through PRRs reflects a strategy of the innate immune system to prevent the generation of escape mutants that would thus lack the ability to cause infection.

Toll-like receptors (TLRs) are among the best-characterized PRRs. By virtue of a conserved intracellular toll/interleukin-1 receptor/resistance (TIR) domain, they were grouped to the interleukin-1 (IL-1)/TLR superfamly, which also comprises receptors involved in inflammatory response, for example, cytokine receptor interleukin-1 receptor (IL-1R). However, toll receptors are further characterized by amino-terminal and extracellular leucine-rich repeats (LRRs; each 20–30 amino acids [aa] in length), whereas the IL-1Rs have extracellular immunoglobulin domains. Apart from the TLR and IL-1R subgroups, the TLR superfamily comprises a third subgroup, cytoplasmic adapter proteins with no extracellular region.

The toll protein was originally described as a type I transmembrane receptor that controls dorsoventral patterning of the Drosophila embryo (Hashimoto et al. 1988). In 1996, Lemaître et al. demonstrated the involvement of the Drosophila toll receptor in the antifungal immunity. Since then, members of the ever increasing toll family have been identified in many other organisms, mostly through expressed sequence tag and genomic sequence database analyses. Thus, toll homologues were discovered in vertebrates, arthropods, and Caenorhabditis elegans (Medzhitov and Janeway 2000; Underhill and Ozinsky 2002; Akira 2003). PAMPs recognized by those TLRs include lipopolysaccharides (LPSs) and lipoproteins (LPs), peptidoglycans, flagellins, dsRNA, distinct nucleic acid motifs, etc. Upon direct or indirect ligation of PAMPs to TLRs the receptors homo- or heterodimerize, consequently triggering various conserved and intracellular signal cascades. In most cases this involves recruitment of cytoplasmic TIR domain–containing adapter molecules to the aggregated receptors, in particular MyD88, which also contains another protein interaction domain, the death domain (DD). Subsequently, MyD88 associates with the DD-bearing serine/threonine protein kinase IL-1 receptor–associated kinase-4 (IRAK-4) through homophilic DD–DD interaction, thus recruiting the kinase to the receptor–adapter complex. Following the activation of IRAK-4 downstream signaling leads to an activation of transcription factors, such as nuclear factor kappa B (NF-κB). Consequently, the expression of costimulatory and regulatory molecules is induced. Also, defense mechanisms that directly oppose foreign invaders are triggered, such as proapoptotic, proinflammatory, and immunomodulatory cytokines, antimicrobial peptides, or even proteolytic cascades that involve the key effectors of apoptotic cell death, the caspases (see: Takeda 2005). However, the reaction elicited often depends on the cell type; in human polymorphonuclear neutrophils, for example, TLR-induced activation of NF-κB

Key words: sponges, innate immunity, toll-like receptor, IRAK, TIR, death domain, caspase, lipoproteins.

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delayed apoptosis through modulation of Bcl-2 proteins (Francois et al. 2005).

Already since 1907, when Wilson (1907) documented the species-specific reaggregation of poriferan cells, sponges became a classical model for the study of cellular self/self- and self/nonself recognition. Because sponges are located near the root of the tree of metazoan life, they turned out to be highly suitable to study the molecular evolution of metazoan innate immunity. With the emergence of molecular biological techniques, a wealth of molecules involved in the poriferan innate immune response was identified. These findings are not surprising if one considers that sponges filter each day huge amounts of water through their aquiferous canal system in search of edible material. Because the aqueous milieu is rich in microorganisms (Bergh 1908; Vacelet et al. 1994). However, not only are Porifera a rich source of bioactive nonproteinaceous metabolites with potential antimicrobial function (Sarma et al. 1993; Proksch 1994; Faulkner 2002), but they are also provided with various proteinaceous molecules of defense that were honed to perfection during 800 MYR of evolution, ensuring the survival of the phylum. Among the poriferan proteins are those of apoptotic cell death (e.g., Bcl-2 and caspases [Wiens and Müller 2006]), antiviral defense (several 2,5’-oligoadenylate synthetases [Wiens et al. 1999; Grebenjuk et al. 2002]), antifungal response (the 1(3)-β-d-glucan-binding protein [Perović-Otštad et al. 2004]), and antibacterial defense. The latter one features receptors of bacteria or bacterial products (Group A/B scavenger receptors [Blumbach et al. 1998; Pahler et al. 1998]), mediators (stress-activated mitogen-activated protein kinases p38 and c-jun N-terminal kinase [Böhm et al. 2000, 2001]), and effector molecules (lysozyme- and tachylectin-related molecules [Thakur et al. 2005]). Furthermore, the poriferan response to gram-negative bacteria was elucidated recently (Wiens et al. 2005). Thus, sponge LPS–interacting protein (SLIP), a transmembrane receptor on the sponge cell surface, was found to recognize and to bind the endotoxin LPS. After dimerization SLIP triggers intracellular signaling through interaction with poriferan MyD88 (myeloid differentiation factor 88), an adapter that binds the endotoxin LPS. After dimerization SLIP triggers intracellular signaling through interaction with poriferan MyD88 (myeloid differentiation factor 88), an adapter that bears 2 protein interaction domains, TIR and DD. Subsequent signal transduction analysis showed that exposure of sponge cells to LPS resulted in an elevated expression of MyD88 and of macrophage expressed gene, a perforin-like executing molecule with antibacterial (gram-negative) effect.

Sponges are filter feeders and thus highly exposed to bacterial invaders. Because LPs are nearly ubiquitous constituents of bacterial cell walls, sponges were screened for the molecular repertoire that is part of the LP-stimulated innate immune response. In the present study, we report on the identification of a TLR, a serine/threonine protein kinase (IRAk-41), and a novel effector caspase in the sponge Suberites domuncula. To underscore the function of these molecules in the antimicrobial defense system, S. domuncula was exposed to Pam3Cys-Ser-(Lys)4, a synthetic triacyl lipopeptide that mimics bacterial LPs. In response to this lipopeptide challenge, TLR and IRAk-41 displayed a constitutive expression. In contrast, the expression of the poriferan effector caspase dramatically increased. This effect could be blocked in competition experiments with recombinant TLR (rTLR). Furthermore, in situ hybridization studies revealed a predominant expression of TLR in the cell layer of the sponge surface and also in cells lining the aquiferous canal system.

Materials and Methods

Chemicals and Enzymes

The sources of chemicals and enzymes used were given previously (Wiens et al. 2005). In addition, (S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH 3HCl (Pam3Cys-Ser-Lys4) was purchased from Axxora Deutschland GmbH (Grünenberg, Germany); natural sterile-filtered seawater (SW) was obtained from Sigma-Aldrich (Taufkirchen, Germany); PCR-DIG-Probe Synthesis Kit, CDP-Star (disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2’-(5’-chloro)tricyclo[3.3.1.1.3,7]decan]-4-yl]phenyl phosphate), 4-nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and anti-DIG Fab fragments conjugated to alkaline phosphatase (anti-DIG-AP) from Roche Applied Science (Mannheim, Germany); TRIZol Reagent, SNAP Total RNA Extraction Kit, and competent TOP10 cells from Invitrogen (Karlsruhe, Germany); and Hybond N+ and antimicrobial antibody from GE Healthcare Europe GmbH (Munich, Germany).

Sponges

Specimens of the marine sponge S. domuncula (Porifera, Demospongiae, Hadromerida) were collected near Rovinj (Croatia) in the northern Adriatic Sea and then kept in aquaria in Mainz (Germany) at 17 °C.

Cloning of the Poriferan TLR SDTLR, the IRAK-41 Protein SDIRAK41, and the Caspase-Like Protease SDCASL

The complete sponge cdNA SDTLR, coding for the putative poriferan TLR_SUBDO, was isolated from a S. domuncula cdNA library (Kruse et al. 1997). A degenerate forward primer, 5’-AAA/G ATT/C/A GTI A/GTI GTI G/C/TTI A/TTI C/TTI (where I = inosine), was designed against a stretch of conserved aa, K-I-V-I/V-V-L/V-S, that is part of the TIR domain (Smart accession number SM00255). In combination with a library-primer-specific primer, polymerase chain reaction (PCR) was carried out at an initial denaturation for 5 min at 95 °C, followed by 35 amplification cycles at 95 °C for 30 s, 54 °C for 45 s, and 74 °C for 1 min, and terminated with a final extension at 74 °C for 10 min. Thus, a fragment of ~400 bp was isolated and subsequently sequenced by means of an automatic DNA sequenator (Li-Cor 4200). Ultimately, the SDTLR sequence was completed through primer walking.

Likewise, the complete poriferan cdNAs SDIRAK-41 and SDCASL were isolated from the S. domuncula cdNA library: application of the degenerate forward primer 5’-CAT/C C/AGI GAT/C A/AGT AA/G T/AC/GI GCI AA-3’ (directed against the IRAk-4 catalytic site H-RD-I/V-K-S-A-N [Prosite Pattern accession number PS00108]) and the degenerate forward primer 5’-T/CTI
ATT/C/A CAA/G GCI TGT/C A/T/C/G TI GG3′ (directed against the caspase Cys active site L/F-I-D-A-C-R/L-G [accession number PS01122]) resulted in fragments of ~700 bp and ~1200 bp, respectively. Again, the fragments were completed by using a combination of sequence-specific and library-specific primers.

Sequence Analyses

Via the servers at the European Bioinformatics Institute, Hinxton, United Kingdom (http://www.ncbi.nlm.nih.gov/BLAST/) and the National Center for Biotechnology Information (NCBI), Bethesda, MD (http://www.ncbi.nlm.nih.gov/BLAST/) homology searches were performed. Multiple alignments were carried out with ClustalW version 1.6 (Thompson et al. 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments applying the Neighbor-Joining method to the distances that were calculated using the Dayhoff PAM matrix model (Dayhoff et al. 1978; Saitou and Nei 1987). The graphical output of the bootstrap figures was produced through the ‘‘Treeview’’ software (R. D. M. Page, University of Glasgow, United Kingdom; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Further graphic presentations were prepared with GeneDoc (Nicholas KB and Nicholas NB 1997). Potential subunits, domains, patterns, and transmembrane regions were predicted after searching the Pfam database (Finn et al. 2006; http://www.sanger.ac.uk/Software/Pfam/), the SMART database (Letunic et al. 2006; http://smart.embl-heidelberg.de/), or according to Kyte and Doolittle (1982).

Preparation of Recombinant TLR_SUBDO

The forward primer 5’-GGATCCACATGTC-CACCTTTTCTT-3’ (BamHI site is underlined) and the reverse primer 5’-GTCGACTACACTCTAACCAGACA C-3’ (SalI site is underlined) were used to amplify the complete open reading frame (ORF) of S. domuncula TLR (nt132–1011), including Metstart and stop codon. This cDNA was ligated in frame into the bacterial expression vector pQE32 (Qiagen, Hilden, Germany). The rTLR protein, tagged with an His stretch, was expressed in TOP10 Escherichia coli cells, extracted with BugBuster Protein Extraction Reagent (Novagen, Madison, WI), and purified through nickel-nitrotriacetic acid (Qiagen) resin treatment (Hochuli et al. 1987). Protein concentrations were determined according to Lowry et al. (1951). Subsequently, the protein was checked by 12% polyacrylamide gels containing 0.05% sodium dodecyl sulfate (Laemmli 1970). Ultimately, after blotting on polyvinylidene fluoride membranes (Millipore, Billerica, MA), the recombinant protein was detected with antihistidine antibodies.

Incubation of Sponge Tissue with Pam3Cys-Ser-(Lys)4

Suberites domuncula tissue samples were exposed to the synthetic lipopeptide (S)-[2,3-Bis(palmitooyloxy)-2-RS-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys3OH·3HCl (Pam3Cys-Ser-(Lys)4) in SW at a final concentration of 60 μg/ml for up to 32 h. In another series of experiments, Pam3Cys-Ser-(Lys)4 was preincubated for 30 min with recombinant S. domuncula TLR (rTLR; 6 mg/ml), prior to the exposition of sponge tissue.

In Situ Localization Studies

The in situ hybridization method applied is based on a procedure described by Polak and McGee (1998) with modifications recently described (Perović et al. 2003). In short, frozen sections (8 μm) of sponge tissue samples (control or Pam3Cys-Ser-(Lys)4-treated) were fixed in 4% paraformaldehyde, then incubated with proteinase K (1 μg/ml), and fixed again in paraformaldehyde. Hybridization was performed overnight at 45 °C in 2 × SSC sodium chloride/sodium citrate (SSC supplemented with 50% formamide), either with sense (control) or antisense probes. The single-stranded DNA probes (230 nt in size) had been labeled with the PCR-DIG-Probe Synthesis Kit through PCR using a part of the TIR domain of SDSL as linearized template. Following hybridization the sections were washed at 50 °C at decreasing salt concentrations (1 × SSC to 0.2 × SSC), blocked, and then incubated with anti-DIG Fab fragments, conjugated to alkaline phosphatase. Through addition of NBT and BCIP, hybridized probes were visualized.

RNA Extraction and Northern Blotting Analyses

Total RNA was isolated through lysis of homogenized and pulverized tissue (control, Pam3Cys-Ser-(Lys)4treated or Pam3Cys-Ser-(Lys)4/rTLR–treated S. domuncula tissue), using TRizol Reagent as described earlier (Grebenjuk et al. 2002). Following purification with SNAP Total RNA Isolation Kit, 5 μg of total RNA each was size separated and blotted on Hybond N+ membrane. Hybridization was performed with a SDSL probe (nt607–1008, encoding the TIR domain), SDIRAK-41 probe (nt199–1070, encoding most of the STYKc domain), or SDCASL probe (nt49–822, encoding the putative p20 and p10 subunits). Furthermore, a probe was designed to detect the expression of the housekeeping gene β-tubulin (TUB) (SDTUB, nt381–481; accession number AJ550806) as reference. Prior to the hybridization the probes had been labeled with DIG-11-deoxyuridine triphosphate through the PCR-DIG-Probe Synthesis Kit (according to the manufacturer’s instructions). Hybridized probes were detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase) and visualized by the chemiluminescence technique using cytidine 5′-diphosphate (CDP) Star as substrate.

Antibody Preparation and Immunohistological Analysis

Polyclonal antibodies (pAbs) were raised against recombinant TLR_SUBDO in female rabbits (White New Zealand) as described (Harlow and Lane 1988; Wimmer et al. 1999). In control experiments, 100 μl of the pAbs were adsorbed to 20 μg of the recombinant protein during an incubation period of 30 min (4 °C) prior to their use. Sponge tissue was fixed in paraformaldehyde, embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), and sectioned, as described (Grebenjuk et al. 2002). Sections of 2 μm were then incubated first with pAbs (1:1000 dilution) and subsequently with Cy3-conjugated goat anti-rabbit IgG (Sigma-Aldrich). The sections were
inspected for immunofluorescence with an Olympus AHB3T microscope. Control experiments with preimmune serum did not show autoimmunofluorescence.

Results and Discussion
Cloning of the Poriferan Toll-Like Receptor SDTLR

The complete SDTLR cDNA was isolated from a S. domuncula cDNA library by means of PCR and a degenerate primer that was directed against a conserved region within the TIR domain of TLRs. SDTLR consists of 1,274 nt (excluding the poly(A) tail) that embrace an ORF of 1,008 nt (excluding the first stop codon), beginning at nt134–137 (Metstart). During northern blot analyses, a DIG-labeled SDTLR probe detected a transcript whose size was consistent with that of the cDNA (∼1,300 nt; fig. 4). The deduced protein was termed TLR_SUBDO and contains 336 aa with an expected size of 37,750 D. TLR_SUBDO shows significant sequence similarity to TLR-1 of Gallus gallus with an expect value (E value; Coligan et al. 2000) of $6 \times 10^{-7}$, but also to human/murine TLR-6 (E value = $3 \times 10^{-6}$/5/$6 \times 10^{-6}$) or human/murine TLR-1 (E value = $5 \times 6^{-6}$, both).

TLRs are characterized by a COOH-terminal intracellular TIR domain, required for the interaction with adapter proteins that equally contain a TIR domain. Furthermore, TLRs bear a transmembrane region as well as several LRRs (2–42 motifs of 20–30 aa in length) in their amino-terminal extracellular part.

Bell et al. (2003) devised a consensus sequence for LRRs of higher Metazoa. Accordingly, LRRs consist of a leucine-bearing portion (consensus: x-L-x(2)-L-x-L-x(2)) required for binding of PAMPs and a helical portion (HP) (consensus: [SNCT]-x-Ω-x(2)-Ω-x(4)-F-x(2)-L; Ω = any hydrophobic residue). However, deviation from these patterns both in length and in sequence is not uncommon among TLRs.

TLR_SUBDO displays 2 of those major characteristics of the TLR superfamily: a TIR domain (aa198–333, E value = $5.4 \times 10^{-6}$; Smart accession number SM002355) and a transmembrane anchor (aa446–66; Kyte and Doolittle 1982). Yet, no LRRs sensu stricto were identified in TLR_SUBDO and the putative extracellular amino terminus (eN-t) is unusually short (43 aa). However, the eN-t of the poriferan TLR has an elevated occurrence of polar or charged aa (65%) and does contain a number of leucine residues as well as valine and serine residues (total of 16). Interestingly, the arrangement of aa revealed a pattern, with [FV]-x(1,2)-[SN]-L 4 times repeated (fig. IA). The number of these repeats is smaller than in TLRs of higher Metazoa and the repeats follow only marginally the consensus pattern of the ligand-binding portion. Nevertheless, after the last repeat the poriferan TLR sequence completely fits the consensus of the HP (fig. IA).

Phylogenetic Analysis of the Poriferan TLR

The toll receptor was initially identified as a protein that is essential for the dorsoventral polarity during Drosophila embryogenesis. Since then, further homologues of the toll receptor (bearing the characteristic TIR domain) have been found not only in Drosophila and other insects but also in vertebrates (at least 10 human TLRs have been characterized, several more in other vertebrates) and in the nematode C. elegans.

Furthermore, TIR domains were also found in several adapter proteins (e.g., MyD88, TRIF [TIR-domain-containing adaptor inducing interferon-beta], and TIRAP [TRIF-related adaptor molecule]) that regulate downstream signaling upon activation of TLRs. In addition, several toll-related proteins that contain TIR have been discovered in plants, where they are implicated in the resistance to pathogens (Jebanathirajah et al. 2002). However, little evidence exists that this limited structural similarity reflects evolutionary conservation of an ancient signaling pathway. There are not only no similarities in downstream signaling pathways activated by metazoan TLR and plant disease resistance proteins, but there is also in general no overlap between immune signaling components of plants and animals (Ausubel 2005).

Interestingly, sequence analyses of the poriferan putative TLR displayed higher aa sequence similarity to vertebrate TLRs than to those of invertebrates, especially regarding the protein interaction domain TIR (fig. IB). Thus, TLR_SUBDO shares 21% identical and 37% similar aa of its TIR with the respective domain of human TLR-1 and chicken TLR-6, 20%/38% with TIR of human TLR-10, and 22%/36% with the murine TLR-6 TIR domain.

To determine the phylogenetic relationship of the poriferan TLR, molecular phylogenetic analyses were performed by the Neighbor-Joining method (Saitou and Nei 1987). Based on the significant similarities between the poriferan protein and TLRs, the TIR domain of TLR_SUBDO was aligned with the corresponding domain of representative members of the metazoan taxa, also including the only other known poriferan TIR domain of the recently discovered MyD88_SUBDO (Wiens et al. 2005). After rooting with the TIR domain of the plant Medicago truncatula TIR/disease resistance protein, the resulting phylogenetic tree revealed 2 clusters (fig. 1C). The first one contains in TLRs, whereas the second one exclusively comprises vertebrate sequences. The latter one is further divided into 5 groups, containing TLR-2, TLR-3/5, TRL-4, TLR-7/8/9, and TLR-1/6/10. The poriferan TLR appears near the root of the phylogenetic tree, at the origin of the aforementioned clusters, closely followed by MyD88_SUBDO. However, the phylogenetic relationship of the C. elegans TIR domain protein family member could not be resolved.

These phylogenetic analyses revealed that the poriferan TLR shares a common ancestor with the TLRs of invertebrates and vertebrates, as indicated by robust bootstrap values. In the course of evolution the ancestral gene likely duplicated several times leading to a family of proteins that has at least 10 members in humans. Because the extracellular LRR domains of TLRs are implicated in recognition and binding of PAMPs (Bell et al. 2003), their subsequent evolutionary diversification probably allowed an enhanced distinction between a vast array of structurally unrelated microbial ligands in higher Metazoa.

Cloning of the Poriferan IRAK-4l Protein SDIRAK-4l

SDIRAK-4l was isolated from a Suberites cDNA library by means of a degenerate primer, designed to target
Suberites domuncula TLR. (A) The deduced poriferan TLR aa sequence of *S. domuncula* (TLR_SUBDO) is depicted. Four extracellular repeats of a leucine-containing motif are shown as well as the HP, the putative transmembrane stretch, and the intracellular TIR domain. (B) The TIR domain of TLR_SUBDO (aa198–333) was aligned with the respective domain of human TLR-10 (TLR10_HOMO; NCBI accession number AA09113; aa633–774), TLR-6 (TLR6_HOMO; BAA78631; aa645–780), and TLR-1 (TLR1_HOMO; NP_003254; aa868–775). *G. gallus* TLR-1 (TLR1_GALLUS; NP_001007489; aa647–788), and murine TLR-6 (TLR6_MUS; AAH53566; aa643–779). Residues conserved (identical or similar with respect to their physicochemical properties) in all sequences are shown in white letters on black; those in 80% are in white letters on gray. (C) Rooted tree generated after alignment of the aforementioned TIR domains, further integrating the TIR domains of human TLR-9 (TLR9_HOMO; NP_059138; aa120–324), TLR-8 (TLR8_HOMO; NP_057694; aa108–1040), TLR-7 (TLR7_HOMO; NP_059746; aa106–1055), TLR-5 (TLR5_HOMO; AAC34376; aa272–278), TLR-4 (TLR4_HOMO; CAH72620; aa174–215), TLR-3 (TLR3_HOMO; AAA9737; aa270–283), TLR-2 (TLR2_HOMO; AAA3756; aa640–784), of *Drosophila melanogaster* TOLL (TOLL_DROME; NP_733166; aa183–320), TOLL-7; (TLR7_DROME; AAF86225; aa111–1245), TOLL-6 (TLR6_DROME; AAF86226; aa111–1243), TOLL-5 (TLR5_DROME; AAF86227; aa163–779), TOLL-4 (TLR4_DROME; AAF86228; aa163–779), TLR-3 (TLR3_DROME; AAF86229; aa163–781), and 18 wheeler (18WHEEL_DROME; NP_478214; aa163–1120), of *Caenorhabditis elegans* TIR domain protein family member (TIR_CAE; NP_478214; aa163–560). of *G. gallus* predicted TLR-4 (TLR4_GALLUS; NP_001035864; aa688–825), of *D. rerio* TLR4b (TLR4b_DANIO; NP_997977; aa668–814), TLR-3 (TLR3_DANIO; NP_997978; aa668–814), TLR-2 (TLR2_DANIO; NP_997979; aa668–814), of *Tribolium castaneum* predicted protein precursor (TLR3_TRICAS; NP_976779; aa37–97), of murine TLR-4 (TLR4_MUS; AAH9856; aa672–813), of *S. domuncula* MyD88 (MyD88_SUBDO; CAI68016; aa114–251), and of the plant *Medicago truncatula* TIR/disease resistance protein (TIR_MEDTRU; ABE93165; aa24–162). The latter one was used as an outgroup. The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence. The numbers at the nodes are an indication of the level of confidence for branching (1,000 bootstrap replicates).
the catalytic site of Ser/Thr/Tyr kinase domain (STYKc domain, Smart accession number SM00221) that generally characterizes IRAks. The complete cDNA comprises 1,254 nt (excluding the poly(A) tail) that contain an ORF of 1,062 nt (nt 40–42[Met start] to nt 1102–1104[Stop]), encoding a putative protein of 354 aa (expected molecular weight [Mr] of 39,619) that was termed IRAK4l_SUBDO. Northern blot analyses confirmed the size of the transcript (fig. 4A).

Phylogenetic Analysis of the Poriferan IRAK-4l Protein

IRAKs constitute a family of serine/threonine kinases, originally identified through coprecipitation of activated IL-1 receptor (Martin et al. 1994). The mammalian IRAK family consists of at least 4 members that mediate the activation of transcription factors (especially NF-κB) and mitogen-activated protein kinases: IRAK-1, IRAK-2, IRAK-4, and IRAK-4l protein of S. domuncula (IRAK4l_SUBDO; aa52–346), putative kinase of Arabidopsis thaliana (PPK_ARATH; AAF99862; aa290–567), and putative receptor protein kinase of Oryza sativa (PPK_ORYZA; XP_550056; aa345–618) that was used as an outgroup.
IRAK-M, with different splice variants that are sometimes highly truncated. However, only IRAK-1, IRAK-4, and Pelle (Drosophila melanogaster IRAK homologue) contain a functional catalytic site within the kinase domain STYKc, with a critical aspartate residue (D187 for human IRAK-4 short-form variant 4). In IRAK-2 and IRAK-M, this critical residue has changed into a serine or an asparagine, rendering their kinase domain inactive (Wesche et al. 1999). Furthermore, IRAK-1, IRAK-2, and IRAK-M but not Pelle or IRAK-4 contain a COOH-terminal region required for interaction with downstream adapter molecules. The recently identified IRAK-4 appears to be the apical kinase in the TLR-signaling pathways (Li et al. 2002). So far, only a limited number of IRAK-4 homologues have been identified in vertebrates and others in insects (D. melanogaster [Pelle] and Apis mellifera [predicted protein kinase]), nematodes (C. elegans [Pelle IRAK-like kinase]), and gastropods (Euprymna scolopes [IRAK]). Again, the poriferan protein shows highest sequence homology to vertebrate proteins, in particular to human IRAK-4 (27% identical and 40% similar aa) and to the murine protein (21%/32%). Also, it comprises the characteristic STYKc domain, with the critical aspartate residue (D187) suggesting an active kinase.

On the other hand, IRAK-4l_SUBDO does not include the amino-terminal DD that is required for homophilic DD–DD interaction with the upstream adapter MyD88. At least one mammalian variant of IRAK-4 (IRAK-4 short-form variant 4; AAR02363) also misses DD, indicating the conservation of this truncated version. How IRAK-4l_SUBDO or IRAK-4 short-form variant 4 are recruited to upstream adapter molecules and thus to the TLRs is currently unknown. The amino-terminal stretch (UD for undetermined region; fig. 2A) that precedes the STYKc domain might be involved in recruitment to adapter proteins that either mediate MyD88-independent signal transduction or facilitate the binding to MyD88.

Because the poriferan IRAK-4l protein is characterized by a truncated amino-terminal part, only the conserved STYKc domain was considered for phylogenetic studies. Given that no plant IRAKs are known, the resulting tree of both clusters, hence forming the basis of the IRAK-4 family (fig. 2B).

Cloning of the Poriferan Caspase–Like Protease SDACS

Caspases (cysteine-dependant aspartyl-specific proteases) are highly selective cysteine proteases that cleave their substrate after an aspartic acid residue that is part of a tetrapeptide motif, hence leading to the dismantlement of the apoptotic cell (Takahashi and Earnshaw 1996; Thornberry and Lazebnik 1998).

After identification of SDACS through degenerate primer PCR, targeting the cysteine catalytic site, a complete clone (1,681 nt, excluding poly(A)) was isolated from the Suberites cDNA library. Subsequently, the correct size of the SDACS transcript was verified on northern blot (fig. 4A). SDACS comprises an ORF between nt148–150 (Metstart) and nt1579–1581 (Stop), coding for a putative protein of 477 aa that was termed CASL_SUBDO (expected Mr of 53475). This protein revealed greatest sequence homologies to caspase-7, especially to human caspase-7 chain A/B (E value = 2 × 10⁻11), but also to Drosophila DCP-1 (7 × 10⁻11) and to caspase-8 of Bos taurus (5 × 10⁻9).

Different caspases have different domain architecture depending on their role in the apoptotic process (Salvesen and Dixit 1997; Thornberry et al. 1997). However, all of them are expressed as zymogens containing the CASc domain (Smart00115). In order to become active as cysteine peptidases, this domain has to be cleaved into a large (p20) and a small (p10) subunit. CASL_SUBDO putatively represents such a proenzyme comprising the 2 subunits, p20 (aa17–152) and p10 (aa184–274) (fig. 3A). As a general rule, p20 harbors the catalytic cysteine, histidine, and glycine residues that are part of conserved patterns. Regarding the caspase family-specific cysteine active site CASL_SUBDO follows the established pattern [K]P-[K]-[LIVMF]-[LIVMF]-[LIVMF]-[2]-[2]-[AF]-C-[RGGL]-[GE] (Prosite Pattern PS01122) with 3 exceptions (marked in bold). However, the sequence homologous to the histidine active site (PS01121) revealed a surprising number of alterations (marked in bold): [HP]-x(2,4)-[SC]-x(2)-[A]-x(0,1)-[LIVMFY]-[2]-[ST]-G-H-G. Thus, CASL_SUBDO bears the cysteine, histidine, and glycine residues that form the catalytic triad. Yet, it also features variations of otherwise conserved positions. This change might impose a novel substrate cleavage specificity for this poriferan caspase. Furthermore, similar to caspase-7 of other species and downstream caspases in general, the Suberites caspase-like protease only has a small amino-terminal prodomain (aa1–16) and does not carry the large prodomain that is characteristic of initiator caspases (see below). Consequently, CASL belongs to the group of downstream caspases. These effector caspases usually cleave key regulatory proteins with a preference of a D/VEXD sequence at the cleavage site of their substrates (Srinivasula et al. 1996; Talianian et al. 1997; Thornberry et al. 1997). However, CASL carries an uncharacteristically large COOH-terminal stretch of 203 aa following the p10 subunit. This segment (aa405–475) revealed no similarity to any published sequence so far except a weak homology (E value = 7.5 × 10⁻²) to a double stranded RNA–binding motif (aa405–475; Pfam accession number PF0035.11).

Phylogenetic Analysis of the Poriferan Caspase–Like Protein

The identification of CED-3, a C. elegans homologue to human mammalian caspase-1, provided the first evidence that the apoptotic cell death machinery is conserved across species (Yuan et al. 1993). Currently, 13 members of the caspase family have been identified in humans alone; at least 3 caspases were predicted to be present in the proteome of the sea urchin Strongylocentrotus purpuratus, and not less than 6 caspases were found in D. melanogaster, 4 in C. elegans, and 4 in sponges (including SDACS).
Furthermore, several proteases that are more closely related to caspases-7 chain B (CAS7B_HOMO; 52095494; aa89-210) and of Drosophila melanogaster caspase DCP-1 (DCP1_DROME; AAB58237; aa77-351) were identified in plants, fungi, and bacteria. They were termed meta- and paracaspases (Uren et al. 2000). Possibly they are also involved in cell death; however, their function remains to be elucidated.

Caspases can be grouped according to their prodomain structure into 2 broad classes (Salvesen and Dixit 1997; Thornberry et al. 1997). Initiator or apical caspases carry a large prodomain that comprises 1 of 2 protein–protein interaction domains, a death effector domain or a caspase recruitment domain. In contrast, effector or basal caspases...
either lack or carry only a short prodomain. Because CASL features an uncommonly large COOH-terminal region and its prodomain consists of not more than 16 aa, for phylogenetic analyses only the large and small subunit–bearing CASc domain was considered to be of informative value. Thus, poriferan CASc was aligned with the corresponding domains of its closest relatives, including humans, chicken, *Xenopus laevis*, *S. purpuratus*, *D. melanogaster*, and *C. elegans* caspases as well as a metacaspase of *Schizosaccharomyces pombe*. The alignment revealed highest aa sequence homology to vertebrate caspase-7, sharing 21% identical and 37% similar aa with the human protein, 21%/35% with the *Xenopus* molecule, and 20%/35% with that of chicken. The homology to other caspases was slightly less, for example, chicken caspase-1 (13%/29%) and human caspase-12 (13%/30%), or considerably lower in case of the yeast metacaspase (8%/20%). A phylogenetic tree, based on this alignment, was rooted with the distantly related *Nitrobacter* Cys protease C14 containing the caspase catalytic subunit p20 (fig. 3B). The resulting tree revealed 6 clusters, comprising caspase-3 and -7 (1), caspase-6 and DCP-1 (2), caspase-8 and -10 (3), caspase-9 (4), caspase-2 and CED-3 (5), and a sixth cluster with a greater diversity, consisting of caspase-1, -4, -5, and -12 and the *D. rerio* caspase-2. No resolution as to the positioning of the yeast metacaspase could be obtained, likely due to the putative mitochondrial origin of metacaspases (Koonin and Aravind 2002). However, the positioning of the poriferan molecule CASL at the origin of the clusters reflects the ancient character of this protease, strongly supporting the monophyly of classic caspases.

**Gene Expression in Response to Pam_{3}Cys-Ser-(Lys)_{4} Treatment**

LPs are found abundantly in both gram-negative and gram-positive bacteria. They represent an important class of PAMPs and are known to induce a broad spectrum of host-defense mechanisms. In particular, the functional association of TLR-1 or TLR-6 with TLR-2 in response to diacyl and triacyl lipopeptides stimulates antimicrobial pathways (Brightbill et al. 1999; Ozinsky et al. 2000; Buwitt-Beckmann et al. 2005). Its direct binding to TLRs induces TLR heterodimerization and mimics the immunomodulatory effects of bacterial LP. Thus, after binding to TLRs, an active signaling complex is formed, including the adapter MyD88 and IRAK proteins, resulting in the production of antimicrobial compounds through the activation of transcription factors (Takeuchi et al. 2001, 2002; Buwitt-Beckmann et al. 2005).

Bacterial LPs and several other PAMPs have also been shown to trigger apoptosis through TLR-mediated signaling. This pathway probably bifurcates at the level of TLR-binding adapters, either via MyD88 and subsequent activation of the caspase cascade or via TRIF and subsequent stimulation of the intrinsic pathway (Aliprantis et al. 1999, 2000; Hsu et al. 2004), consequently resulting in an activation of the caspase cascade.

Furthermore, bacterial LPs mediate activation of NF-κB. This transcription factor has also been implicated in the expression of both pro- and antiapoptotic genes (Baichwal and Baueerle 1997; Wang et al. 1998), complementing the activation of pro- and antiapoptotic proteins on the transcriptional level.

In order to examine the effect of LPs on the expression of these proteins that are also putatively involved in poriferan antimicrobial mechanisms and because the poriferan TLR revealed closest sequence homology to the vertebrate TLR-1 and TLR-6, sponge tissue was incubated with Pam_{3}Cys-Ser-(Lys)_{4} for the indicated time, RNA was isolated, size separated (equal amounts of RNA were loaded), and blotted. The blots were probed for transcripts of poriferan TLR, IRAK-4l protein, CASL, and TUB. The latter one was used as an internal control. (A) Pam_{3}Cys-Ser-(Lys)_{4} was pretreated with rTLR before it was applied to sponge tissue. After incubation of sponge tissue, RNA was isolated and again submitted to northern blot analyses.

![Fig. 4.—Pam_{3}Cys-Ser-(Lys)_{4}–induced gene expression in *S. domuncula*. (A) Following incubation of sponge tissue with the synthetic LP Pam_{3}Cys-Ser-(Lys)_{4} for the indicated time, RNA was isolated, size separated (equal amounts of RNA were loaded), and blotted. The blots were probed for transcripts of poriferan TLR, IRAK-4l protein, CASL, and TUB. The latter one was used as an internal control. (B) Pam_{3}Cys-Ser-(Lys)_{4} was pretreated with rTLR before it was applied to sponge tissue. After incubation of sponge tissue, RNA was isolated and again submitted to northern blot analyses.

In a second set of experiments, Pam_{3}Cys-Ser-(Lys)_{4} was pretreated with recombinant TLR_SUBDO, the putative receptor of bacterial LPs. Only after this “blocking,” the synthetic LPs were applied to sponge tissue for 8 h, 16 h, and 32 h. After RNA isolation, electrophoresis, and blotting, the respective transcripts were identified by means of labeled probes, detecting the poriferan TLR, IRAK-4l protein, caspase-like protease, or TUB (the expression of this housekeeping gene was used as an internal control). The blotting experiments revealed a relatively high level of expression of TLR and IRAK-4l in the control tissue. Regardless of a Pam_{3}Cys-Ser-(Lys)_{4} challenge (up to 32 h), the steady-state level of transcription of those genes remained unchanged. In contrast, transcripts of the CASL were not detectable in control tissue. However, after 8 h of Pam_{3}Cys-Ser-(Lys)_{4} treatment, the CASL expression dramatically increased (fig. 4A).

In a second set of experiments, Pam_{3}Cys-Ser-(Lys)_{4} was pretreated with recombinant TLR_SUBDO, the putative receptor of bacterial LPs. Only after this “blocking,” the synthetic LPs were applied to sponge tissue for 8 h, 16 h, and 32 h. RNA of tissue thus treated was again submitted to northern blot analyses. The resulting blot revealed no Pam_{3}Cys-Ser-(Lys)_{4}–induced expression of CASL. The
transcript level in treated samples was comparable to the steady-state level of expression of control samples (fig. 4B).

These results have several major implications. First, LPs are recognized by sponge cells and trigger the activation of transcription factors. Second, because a caspase is among those proteins whose expression is stimulated, apoptosis takes part in the antibacterial response. Third, LPs blocked with TLR were not able to induce the transcription of CASL, consequently implying a potential role of TLR as a PRR of LPs. Fourth, TLR and IRAK-4 are constitutively expressed, thus allowing an immediate response to microbial threats, which is an imperative prerequisite of innate immunity.

These results taken together lend further proof to the concept that induction of apoptosis by TLRs is an evolutionarily conserved mechanism for innate immune defense. The relevance of TLR-mediated apoptosis, however, remains elusive, though the TLR-induced death of immune effector cells could control the duration of acute response to microbial assaults (Aliprantis et al. 2000).

Localization of TLR-Expressing Cells within Sponge Tissue

Toll-like receptors are transmembrane proteins expressed in cells that are exposed to the environment. Some TLRs are located on the cell surface, whereas others remain sequestered in intracellular compartments (Akira et al. 2006). According to the sequence analyses, S. domuncula TLR displays the prerequisites for recognition of microbial invaders and subsequent innate immune response: an amino-terminal ectodomain–containing 4 leucine–bearing repeats, a transmembrane region (of 23 hydrophobic aa), and a COOH-terminal intracellular TIR domain. To further support the assumption that TLR_SUBDO represents a PRR and thus is part of the frontline, antimicrobial defense of sponges, in situ hybridization analyses were performed. For this purpose, sponge tissue cryosections were incubated with labeled sense or antisense probes of TLR.

Hybridized probes were then detected via successive treatment with anti-DIG-AP antibodies and substrate. Although the sense probe did not produce any signal (fig. 5A), the antisense probe abundantly detected transcripts found in the surface cell layer of the pinacoderm and to a lower degree also in cells lining the aquiferous canal system within the mesohyl, the internal part of the sponge (fig. 5B).

In a parallel set of experiments, tissue sections were treated with pAbS raised against recombinant TLR_SUBDO. The resulting immunocomplexes were then detected with Cy3-conjugated secondary antibodies. These immunohistological analyses revealed again a TLR expression especially favoring the cells of the animal surface (fig. 6A and B) and the canal system (fig. 6C and D).

Thus, TLR is primarily expressed at the interface with the environment and meets another prerequisite of TLRs.

Conclusion

It was Metchnikoff (1892) who first demonstrated the existence of innate immunity in sponges, primarily based on phagocytosis by sponge archaeocytes. With the introduction of molecular tools 100 years later, molecular biological and cell biological support for the presence of this immune system came from studies with the demosponge S. domuncula and Geodia cydonium. The studies clearly revealed that these animals, the closest relatives to the common ancestral phylum Urmetazoa (Müller 2001), are provided with key molecules involved in immune signaling pathways (see Introduction). Interestingly, only in some protostomian taxa, for example, D. melanogaster, the TLR-dependent control of innate immunity could be demonstrated. In contrast, the only C. elegans toll receptor homologue known so far does not appear to play such a role (Pujol et al. 2001). Thus, the results summarized in this
study, which demonstrated that a TLR-mediated antimicrobial defense in sponges exists, came unexpected. This is even more remarkable because—until now—also in the diploblastic Cnidaria a similar TLR has not been described.

In the present work, it is demonstrated that a poriferan TLR-like molecule (TLR_SUBDO), particularly localized in cells on the surface of the animals and in cells lining the aquiferous canal system, mediates LP-stimulated signal pathways, in which the IRAK-4l protein (IRAK4l_SUBDO) is potentially implicated. The constitutive expression of both proteins ensures an immediate response to bacterial challenge that ultimately leads to an increased expression of the caspase-like molecule CASL_SUBDO and subsequent initiation of the caspase-dependent apoptotic machinery. Competition experiments between the soluble recombinant sponge TLR and a synthetic lipopeptide further support the conclusion that this receptor is indeed involved in the innate immune defense of sponges.

TIR domain proteins and caspase-like proteins have been found in plants. Thus, the finding that the synthetic lipopeptide Pam_Cys-Ser-(Lys)4 elicits caspase induction in cells on the surface of the animals and in cells lining the aquiferous canal system, mediates LP-stimulated signal pathways, in which the IRAK-4l protein (IRAK4l_SUBDO) is potentially implicated. The constitutive expression of both proteins ensures an immediate response to bacterial challenge that ultimately leads to an increased expression of the caspase-like molecule CASL_SUBDO and subsequent initiation of the caspase-dependent apoptotic machinery. Competition experiments between the soluble recombinant sponge TLR and a synthetic lipopeptide further support the conclusion that this receptor is indeed involved in the innate immune defense of sponges.

To conclude, these data demonstrate that sponges have a receptor-mediated antibacterial defense system, closely linked to the apoptotic cell death–executing system. The concerted action of these mechanisms, in combination with the previously described LPS-induced signaling pathways and the defense systems against fungi and virus (for references: see Introduction), granted a successful survival of the poriferan taxon throughout metazoan evolution.

Supplementary Material

The following cDNA sequences from *S. domuncula* that have been deposited (EMBL/GenBank) as TLR (SDTLR; accession number AM392428), IRAK-4l Protein (SDIRAK-4l (AM392429), and the CASL (AM392430) are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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Evolutionary Origin of Toll-Like Receptors in Metazoa


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