SUMMARY
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Objective 1: Molecular characterization of rat lysozyme like proteins \textit{in silico}

Using \textit{in silico} approaches, we identified six rat Lyzl genes namely Lyzl1, Lyzl3, Lyzl4, Lyzl5, Lyzl6 and Lyzl7. Lyzl4 sequence was reported to GenBank. \textit{In silico} studies showed that LYZL proteins are similar to c-type lysozyme. Lyzl genes were found to be highly conserved among the vertebrates and are highly homologous to mouse counterparts than with human counterparts. All the LYZL proteins possess the characteristic 4 disulphide bridges similar to that preserved in c-type lysozyme. These proteins are very closely related to each other in sequence similarity, biochemical and structural aspects. LYZL proteins are amphipathic and secretory in nature. Although they are very similar to lysozyme, except for LYZL 1 and 6, the remaining proteins do not conserve the active site amino acids. Molecular modeling studies indicated that LYZL proteins exhibit strikingly similar three dimensional structures among themselves. Docking studies indicated the peptidoglycan binding nature of LYZL proteins. Presence of active site and peptidoglycan binding ability of LYZL1 and 6 suggest that they may be involved in antibacterial mechanism.

Objective 2: Expression profiling and biochemical characterization of rat lysozyme-like proteins

All the rat Lyzl mRNA transcripts (Lyzl1, Lyzl3, Lyzl4, Lyzl5, Lyzl6 and Lyzl7) are predominantly expressed in testes though some of them are expressed in tissues other than reproductive tract. Their expression is androgen independent. The rat LYZL proteins are localized in the germinal epithelium and on the spermatozoa. Recombinant LYZL1 and 6 possessed muramidase, isopeptidase and antibacterial activities, whereas the remaining
proteins did not, which may be attributed to the absence of active sites. The mechanism of antibacterial action of LYZL1 and LYZL6 involved bacterial membrane damage and leakage of cellular contents. Only LYZL1 and 6 possess peptidoglycan binding ability, whereas LYZL3, LYZL4 and LYZL5 possess hyaluronan binding ability suggesting a possible functional divergence of these proteins. In addition LYZL3, LYZL4 and LYZL7 possessed free radical scavenging property, suggesting that they may act as antioxidants. The secondary structure analysis of the recombinant LYZL proteins indicated the presence of α-helix, β-sheet and random coil with α-helix being the majority.

**Objective 3: Functional characterization of rat lysozyme-like proteins**

The role of LYZL proteins in sperm function was studied by neutralizing these proteins on sperm surface using specific antibodies. *In vitro* neutralization of LYZL4 did not affect capacitation or acrosome related events. Whereas, neutralization of LYZL6 showed an inhibitory effect on calcium influx during capacitation and acrosome reaction. Treatment of spermatozoa with LYZL4 or 6 recombinant proteins did not influence capacitation or acrosome reaction. The importance of LYZL proteins in germ cell production and maturation *in vivo* was studied using an auto-antigen model generated by injecting recombinant rat LYZL6 into rats. Antibody against LYZL6 was detected in male reproductive tissue fluids confirming the passage of antibody through the blood testes barrier. In the immunized rats, the male reproductive tissue architecture was not affected but, resulted in decreased sperm count. Further, decrease in sperm motility parameters was also observed. LYZL6 immunized animals showed decreased fertility depicting the potential role played by this protein in male reproduction.
Identification of Toll-Like Receptors in the Rat (Rattus norvegicus): Messenger RNA Expression in the Male Reproductive Tract Under Conditions of Androgen Variation

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Keywords
Androgens, castration, epididymis, Toll-like receptor

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Problem
Although the majority of Toll-like receptors (TLRs) are reported in many species, some of them are not yet described in the rat. Further, factors that govern Tlr expression in the male reproductive tract have received little attention. We attempt to identify and characterize Tlrs in the rat and determine the expression profile under conditions that affect male reproductive tract gene expression.

Method of study
Rat Tlr5, Tlr10, and Tlr11 transcript sequences were submitted to GenBank and in silico characterization carried out using bioinformatics tools. RT-PCR analyses using gene specific primers for rat Tlr1–13 were carried out with RNA isolated from reproductive tract tissues of various experimental groups.

Results
Tlr5, Tlr10, and Tlr11 identified in this study share features that are characteristic of the known TLRs. Abundant Tlr expression was observed in the male reproductive tract of adult and developing rats. Further, Tlr expression was also observed in the epididymides of androgen ablated rats.

Conclusion
Tlr5, Tlr10, and Tlr11 are ubiquitously expressed in the rat. Tlrs seem to be expressed during male reproductive tract development and under conditions of androgen ablation, suggesting the preparedness of the male reproductive tract to detect an infection under all conditions of androgen status.

Introduction
The epithelial surfaces of many organs such as the respiratory, reproductive, and digestive systems are exposed to the external environment and are constantly under threat from invading pathogenic microorganisms. Innate immune mechanisms that exist in the body counter these microbial infections. In most organ systems, the presence of complex infection-driven signaling pathways is very well established. The ability of innate systems to recognize and respond to these attacks is largely mediated by a family of type I transmembrane receptors called Toll-like receptors (TLRs).¹² They are primarily
expressed on many cell types and have the ability to discriminate and recognize distinct microbial components. Recognition of microbial products by TLRs triggers a variety of signal transduction pathways that differ in nature, magnitude and duration of the inflammatory response. The TLR family consists of 13 members (TLR1–13) that are widely expressed in most of the vertebrate species. Among them TLR1–10 have been identified in humans and the remaining three are confined to rodents. Although the majority of TLRs are reported in many species, TLR5, TLR10, and TLR11 have not been reported in the rat.

Each TLR has been shown to recognize specific components of pathogens. For example, TLR4 recognizes lipopolysaccharide, whereas TLR2 is specific for microbial lipopeptides such as peptidoglycan, lipoteichoic acid, and porins. Recognition of specific cell wall components by TLRs triggers a cascade of events involving a variety of adaptor proteins and protein kinases, finally resulting in the activation of immune response genes. Genes that are targeted include those encoding cytokines such as tumor necrosis factor-alpha, interleukin-12p40, interferon-beta, chemokines, adhesion molecules, acute phase proteins, antimicrobial peptides, inducible nitric oxide synthase, and cyclooxygenase 2. Production of pro-inflammatory cytokines during an inflammatory response is very important for mediating the initial host defense against invading pathogens and opportunistic organisms. They collectively provide immediate protection for hosts and induce the development of adaptive immune responses as well.

Infections of the male reproductive tract can pose a threat to normal reproductive and endocrine functions. Epididymitis, a serious clinical condition characterized by inflammation and obstruction of sperm movement, results from the retrograde extension of micro-organisms from the vas deferens. Infection of the epididymis can lead to the formation of an epididymal abscess. In addition, progression of the infection can lead to involvement of the testicle, causing epididymoorchitis or a testicular abscess.

In the recent past, mechanisms involved in the innate immune responses of the male reproductive tract have become an active area of investigation. For example, the expression of antimicrobial proteins and peptides including defensins has been extensively characterized in the male reproductive tract. The expression of lipopolysaccharide-binding protein, an acute phase protein known to play a central role in defense against Gram-Negative bacteria, was demonstrated in the human epididymis. Further, CD14, a 54 kDa glycolipid-anchored membrane glycoprotein, expressed on myeloid cells, which functions as a member of the LPS receptor complex, was demonstrated in the seminal plasma and also on the sperm membranes. A recent study reported the presence of Tlr1–11 and some of their adaptors in the male reproductive tract of rats. Although, majority of TLRs are reported in the rat, TLR5, TLR10, and TLR11 are not. Further, TLR12 and TLR13 have not been characterized in the male reproductive tract of this species. Hence, in silico and in vivo analyses were carried out in this study to identify and characterize Tlr1–11, Tlr10, and Tlr11. Further, the male reproductive tract specific expression profiles of all previously reported Tlrs besides Tlr12 and Tlr13 in the rat were also studied. Gene expression in the male reproductive tract is dependent on tissue levels of androgens that are known to change during development. As very little is known about the factors (such as androgens) that may affect Tlr expression in the male reproductive tract, attempts were made to characterize the expression profile of all known Tlrs under conditions of androgen variation. In this study, we identified and characterized three Tlrs, namely, Tlr5, Tlr10, and Tlr11 in the rat. Further, we demonstrate that majority of the Tlrs analyzed are abundantly expressed in the male reproductive tract of adult and developing rats and also under conditions of androgen ablation.

Materials and methods

Genomics

Using mouse Tlr5, Tlr10, and Tlr11 sequence, the rat genome (build RGSC v3.4) was searched using the BLAST program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to identify the rat orthologs. Five to six sets of intron spanning primers (Table S1) were designed for each Tlr mRNA so that the entire sequence can be amplified in parts. RT-

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PCR was performed using rat epididymis mRNA as the template. The specific products were sequenced, aligned and deposited in GenBank. The corresponding exon/intron boundaries were determined by aligning the cDNA with the genomic sequence. The sequences were translated using the tools available at ExPASy proteomics server (http://ca.expasy.org/tools/dna.html). In silico, domain analyses were carried out using the InterProScan, a signature recognition search against the integrated resource of protein domains and functional sites (http://www.ebi.ac.uk/Tools/InterProScan). The physical and predicted features of the deduced amino acid sequences were analyzed using tools available at Expasy Proteomics Server (http://ca.expasy.org/).

**Tissue Specimens and RT-PCR**

Wistar rat (aged 60–90 days; $n = 3$) tissues were obtained commercially (Zivic Laboratories Inc, Pittsburgh, PA, USA). Prior to shipping on dry ice, tissues were placed in RNAlater (Ambion Inc, Austin, TX, USA) solution overnight at 4°C to allow penetration and fixation. Upon arrival, tissues were immediately stored at −70°C. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 0.5 μg of oligodT (Invitrogen) according to the manufacturer’s instructions. A total of 2 μL of the resultant cDNA was amplified by PCR using gene specific primers (Table I) for Tlr1–13. The number of cycles to amplify each cDNA in the linear range was determined by preliminary PCR under the following conditions: 94°C for 1 min followed by 25–35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and with a final round of extension at 72°C for 10 min. Thrs were amplified using gene specific primers (Table I) for 28–32 cycles and glyceraldehyde phosphate dehydrogenase (Gapdh) for 28 cycles. PCR amplified gene products were analyzed by electrophoresis on 2% agarose gels. Gapdh expression was used as the internal control. To study the androgen regulation of Thr transcripts, epididymides were obtained from sham operated, castrated and testosterone supplemented Wistar rats ($n = 5$ in each group). Testosterone supplementation was supplied by a 20 mg dihydrotestosterone pellet implanted subcutaneously immediately after castration. All the animals were killed 14 days after castration. All procedures involving animals were performed in accordance with the Guiding Principles in the Care and Use of Animals established by the National Institutes of Health and approved by the Institutional Committee on the use of Animals in Research and Education. For studies on the developmental regulation of Thr, epididymides from 10- to 60-day-old Wistar rats, one rat for each age, were obtained commercially (Zivic Laboratories Inc).

**Results**

Tlr5, Tlr10, and Tlr11 transcripts were discovered on the rat chromosome 13, 14, and 15. The rat Tlr5, Tlr10, and Tlr11 cDNA sequences were submitted to GenBank and were assigned the accession numbers FJ750588, FJ755908, and FJ539013 respectively. Expression sequence analysis revealed that Tlr5 was localized at 13q26 region (Fig. 1a), whereas Tlr10

**Table I** Gene Specific Primers Used in This Study

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<th>Primer name</th>
<th>Sequence</th>
<th>Amplicon size</th>
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<td>Gapdh F</td>
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<td>Gapdh R</td>
<td>5’-AGA CAC GGG GAC ATG AGG ACT-3’</td>
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was localized at 14p11 (Fig. 1b) and \textit{Tlr}11 is present at 15p14 (Fig. 1c). \textit{In silico}, protein translation analyses revealed that single exon encodes the full-length protein. The alignment of rat genomic and protein \textit{Tlr}5, \textit{Tlr}10, and \textit{Tlr}11 sequences are shown in Fig. S1. Further analyses reveal that the predicted amino acid sequences of the three TLRs contain the leucine rich repeat (LRR) and Toll/interleukin-1 receptor (TIR) domains, characteristic of the known TLRs (Fig. 2). TLR5 in the rat is predicted to have five LRRs whereas TLR10 and TLR11 contain three LRRs each. Similar to the known TLRs, there is a single TIR domain, a signal peptide and a transmembrane domain in the rat TLR5, TLR10, and TLR11. Further, the cysteine-rich flanking region (CRFR) was found to be present on the C-terminal side of rat TLR5 and TLR10, whereas the same is absent in the rat TLR11 (Fig. 2). The general physical features of the TLRs identified in this study are given in Table II.

\textbf{Fig. 1} Rat \textit{Tlr} localization on chromosomes 13, 14, and 15. (a) \textit{Tlr}5 on chromosome 13; (b) \textit{Tlr}10 on chromosome 14; (c) \textit{Tlr}11 on chromosome 15. Arrows indicate direction of transcription. Positions were taken from the MapView (build 3.4) at the National Center for Biotechnology Information (NCBI) website.
The male reproductive tract is generally a sterile environment and is not routinely exposed to pathogens as are the respiratory and gastrointestinal tracts. However, an exposure to pathogens would be expected to mount an immediate immune response to prevent any damage to the male reproductive tract and to fertility. To determine whether the male tract constitutively expresses the toll-like receptors of the innate immune machinery, the expression of Tlr1–13 mRNAs was analyzed. Majority of the Tlr mRNA transcripts (Tlr2, Tlr4, Tlr5, Tlr6, Tlr7, Tlr8, Tlr10, and Tlr11) were found to be abundantly expressed in all tissues of the male reproductive tract (Fig. 3). However, Tlr1 and Tlr12 expression was only weakly detected in the male tract. Tlr3 expression was mostly restricted to the caput with minimal expression in corpus, cauda, and testis (Fig. 3). Similarly Tlr9 expression was primarily in the seminal vesicle (Fig. 3). To determine whether Tlr5, Tlr10, and Tlr11 are ubiquitously expressed, RT-PCR was performed in a variety of tissues obtained from male and female rats. Tlr5, Tlr10, and Tlr11 were found to be expressed in the non-reproductive as well as the female reproductive tract tissues in the rat (Fig. 4).

Gene regulation in the male reproductive tract is androgen dependent, which in turn varies with the developmental stage of the animal. To determine whether the innate immune machinery is regulated during the course of development, Tlr expression was analyzed in the epididymis, testis, and seminal vesicle.

**Table II** Predicted General Characteristic Features of Rat TLR5, TLR10, and TLR11

<table>
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<tr>
<th></th>
<th>No. of amino acids</th>
<th>Molecular weight (Da)</th>
<th>Isoelectric point (pI)</th>
<th>Glycosylation sites</th>
<th>Phosphorylation sites</th>
<th>Myristilation sites</th>
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<tbody>
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<td>105, 153, 165, 171, 184, 310, 464, 578, 605, 626, 701</td>
<td>35, 78, 130, 202, 252, 266, 321, 545, 567, 751</td>
</tr>
</tbody>
</table>
vesicle of 10- to 60-day-old rats. In the epididymis, as seen in the adult rat, Tlr1 seems to be not expressed abundantly during the course of the development (Fig. 5). The presence of Tlr2, 4, 5, 6, 7, 8, 10, 11, and 12 mRNA was observed at all the stages during development (Fig. 5). The expression of Tlr3, 9, and 13 was detected only in the epididymides obtained from 20-, 50-, and 60-day-old rats. An interesting feature is the presence of all the Tlr mRNA transcripts in the epididymides of 20- and 50-day-old rats (Fig. 5), suggesting a possible role for TLRs at 20- and 50-day time points during development, besides their role in innate immunity.

In the testis, Tlr5, 7, 8, 10, 11, and 12 were found to be abundantly present in all the states during development (Fig. 6), whereas Tlr1, 2, 4, 6, 9, and 13
were weakly expressed. Tlr3, although weakly expressed in the adult rat testes, was not detected in the testes of developing rats (Fig. 6).

In the seminal vesicle, Tlr5, 7, 8, 10, and 11 were found to be abundantly expressed at all the stages of development. However, Tlr1, 4, 6, and 9 expression was weak during the course of development (Fig. 7). Tlr2 and 12 expression was detected in the later part of development, namely, in the seminal vesicles of 30- to 60-day-old rats. On the contrary, Tlr13 mRNA expression was detected up to mid stage of development, i.e. in the seminal vesicles of 10- to 40-day-old rats. Tlr3 mRNA expression was not detected in the seminal vesicles during the course of development.

To develop our understanding of the relation between androgen levels and immune receptor expression, Tlr mRNA levels were analyzed in the epididymides of rats that were either castrated or castrated and supplemented with testosterone (Fig. 8). Interestingly, Tlr1 and 13 mRNA expression was detected abundantly in the castrated rats, whereas the same was weak in the sham operated and testosterone supplemented groups. Abundant expression of Tlr2, 5, 6, 7, 8, 9, 10, 11, and 12 and weak expression of Tlr3 and Tlr4 was detected in all the three groups suggesting an androgen independent expression (Fig. 8).

**Discussion**

TLRs, which recognize the molecular patterns of pathogenic organisms, have been well characterized in many species. However, in the rat, not all have been characterized, and in this study we report the identification of rat Tlr5, Tlr10, and Tlr11 transcripts.
The presence of characteristic LRR and TIR domains in the predicted amino acid sequences of these TLRs suggests that they primarily function to detect pathogens thus sharing the functional features with the known TLRs.

Unlike the respiratory and digestive systems, which are constantly exposed to environmental agents including the pathogens, the male reproductive tract is sterile. Hence, this organ system should be able to initiate a heightened immune response to an infection. Studies on the role and the mechanism of action of innate immune molecules in protecting the male reproductive tract provide a basis for development of new strategies to treat sexually transmitted diseases. Toll-like receptors which form an important component of innate immunity have been implicated in many roles in the male and female reproductive tracts. In the male reproductive tract, TLRs appear to play a role in the control of testicular steroidogenesis and spermatogenesis both in normal and disease conditions. The etiology of prostatitis and prostatic cancer seems to involve TLRs. They have been shown to have a functional role in immune surveillance in the ovary, endometrium, uterus, and placenta. Further, they are involved in ovarian cancer, pelvic inflammatory disease, intrauterine growth restriction, pre-eclampsia, and preterm birth. A recent study demonstrates the abundance of TLRs in the male reproductive tract of rats. However evidence is lacking in describing the abundance of TLRs in general in the male reproductive tract. Such evidence can provide insights into the role of these innate immune molecules during an infection. This study demonstrates the identification of three TLRs namely TLR5, TLR10, and TLR11 in the rat. Further, the expression profile under conditions of androgen variation was also demonstrated, which was not reported in a previous study, wherein the presence of TLRs in the male reproductive tract of rats was analyzed.

In this study, the abundant expression of the majority of TLRs suggests the preparedness of the male reproductive tract to respond to an infection. The presence of other innate molecules such as CD14 and MYD88 in the male tract along with the TLRs strongly points to the fact that invasion by a pathogen might result in the activation of immune effector pathways that can trigger the production of cytokines and antimicrobial peptides. However, further studies are required to demonstrate the execution of such pathways and the repertoire of factors involved in the male reproductive tract. Abundant TLR3 expression was reported in contrast to the weak expression of TLR11 in the male reproductive tract. However, by contrast we observed an abundant TLR11 and weak TLR3 expression. This discrepancy could be because of age and strains of the animals used. Further, male reproductive tract tissues analyzed in this study contain different cell types including the immune cells. It is possible that the expression of TLRs in male reproductive tract tissues could partly be contributed by the immune cells.

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Developmental regulation of a wide variety of genes in the male reproductive system has been studied extensively because of the fluctuations of androgens at various stages. For example, rat epididymis tissue androgen decreases from birth until 20 days, but remains at a substantial level of approximately 10 ng/g tissue (35 nm) until approximately 40 days when it begins to increase to adult levels of between 15 and 20 ng/g. Serum testosterone levels in the rat remain low and do not begin to increase to adult levels until 35–40 days. The expression pattern of majority of Tlrs in the epididymis did not seem to correlate with the minimal androgen levels from day 20 to day 40 or increased androgen in the adult, suggesting that Tlr expression is not androgen dependent during development. However, androgen concentration differs in the testis compared with the epididymis. A steady increase in testosterone levels was reported in the rete testis of 30- to 130-day-old rats. In this study, abundant expression of Tlrs was detected in the testes of 10- to 60-day-old rats suggesting their constitutive nature of expression, thereby keeping the testes ready for any microbial challenge. Lack of Tlr3 expression in the testes of developing rats (10–60 days old) is in contrast to its presence in the testes obtained from adult rats (60–90 days old). Conversely, Tlr10, 11, and 12 mRNA were detected in the testes developing rats (10–60 days old), whereas their expression was not detected in testes obtained from adult rats (60–90 days old). These discrepancies could be because of the age differences and actual levels of testicular testosterone at the time of tissue collection. Further analyses are required to determine whether Tlr3, 10, 11, and 12 expression could be drastically affected within a short window of time. Earlier studies indicate that androgens are important to the development and physiology of the prostate and seminal vesicle. In the seminal vesicles of 10- to 60-day-old rats, Tlr expression was detected at all ages analyzed similar to the testes. Although testosterone is required for development, further studies are required to determine the role of testosterone on Tlr expression during development.

In conclusion, we report the identification of Tlr5, Tlr10, and Tlr11 in the rat. The mRNA expression of Tlrs is abundant in the male reproductive tract of adult and developing rats. Their expression was also detected in the male reproductive tract of androgen ablated rats.

Acknowledgments

This work was supported by Department of Science and Technology, Government of India, New Delhi, India.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Alignment of rat *Tlr* genomic and protein sequences.

**Table S1.** Primers Used to Amplify Rat *Tlr5*, *Tlr10*, and *Tlr11*.

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The human male reproductive tract antimicrobial peptides of the HE2 family exhibit potent synergy with standard antibiotics

Suresh Yenugu* and Ganapathy Narmadha

Reproductive tract infections pose a serious threat to health and fertility. Due to the emergence of antibiotic resistant pathogens, antimicrobial proteins and peptides of the reproductive tract are extensively characterized in recent years toward developing newer strategies to treat genital tract infections. Pathogen growth inhibition using a combination of naturally occurring male reproductive tract antimicrobial peptides and commonly used antibiotics has not been reported. Checker board analyses were carried out to determine the nature of interaction (synergistic, additive and antagonistic) between HE2α and HE2β2 peptides and the commonly used antibiotics. Using Escherichia coli as the target organism, the minimal inhibitory concentration and fractional inhibitory concentration indices were determined. We demonstrate for the first time that the human male reproductive tract antimicrobial peptides HE2α and HE2β2 act synergistically with the commonly used antibiotics to inhibit E. coli growth. A combination of HE2α and HE2β2 peptides resulted in an additive effect. Interestingly, the synergistic effects of HE2 peptides were highest with doxycycline and ciprofloxacin, antibiotics generally used to treat epididymitis. Results of this study demonstrate the potential of endogenous HE2 peptides to be pharmacologically important in designing novel strategies to treat reproductive tract infections. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: epididymis; antimicrobial; synergy; fractional inhibitory concentration index

Introduction

Antimicrobial proteins and peptides are widely expressed in both plants and animals. A variety of natural antibiotics belonging to different classes such as defensins, cathelicidins, cecropins and protease inhibitors [1] are found in epithelial tissues of organs that are exposed to the external environment. Among them, well characterized in humans are the defensins, which are broadly classified into three types, viz. alpha, beta and theta defensins depending on their disulfide bonding, tissue distribution and genomic organization. They exhibit broad spectrum antimicrobial activity [2–5], thus forming an important component of the innate immune system. Antimicrobial proteins and peptides including defensins are generally cationic in nature [6] and are believed to exert their bactericidal effect by permeabilizing the bacterial membranes [7], thinning the membrane [8] or by destabilizing the membrane bilayer [9]. In addition to these effects, antimicrobial proteins and peptides kill bacteria by inhibition of macromolecular biosynthesis [10–12] and/or interacting with specific vital components inside the bacteria [13,14].

In the epididymis, a major organ of the male reproductive tract, immature sperm released from the testis develop forward motility and fertilizing ability as a result of a series of sequential maturation steps. A wide variety of proteins including antimicrobial proteins released into the lumen of epididymis bind sperm and are thought to play an important role in epididymal immunity in addition to their role in sperm maturation [15]. Examples of antimicrobial proteins reported in the male reproductive tract include human cationic antimicrobial protein (hCAP18, a cathelicidin) [16], defensins [17–20], the epididymal β-defensin member Bin1b [21], cystatins [22,23], lactoferrin [24] seminalplasmin [25], seminogelin-derived peptides [26] and members of the HE2 family [27]. The HE2 gene located on chromosome 8p23 within the β-defensin gene cluster encodes a series of isoforms containing identical proregions joined to different C-terminal peptides [27]. Among them, HE2β1 conserves the characteristic β-defensin-like six-cysteine motif. Furthermore, like the β-defensins, HE2 C-terminal peptides are cleaved from their proregions by a furin-like proprotein convertase and these peptides are reported to exist in the epididymal epithelium, luminal fluid and the seminal plasma [28]. We previously identified and characterized an epididymis specific novel defensin, DEFB118, which also conserves the characteristic six-cysteine motif [29]. The antimicrobial activity of HE2α, HE2β1 and HE2β2 proteins and their C-terminal peptides against E. coli [30] and HE2α against Neisseria gonorrhoea, Staphylococcus aureus and Enterococcus faecalis [31] was previously demonstrated. Their antimicrobial activities are structure dependent, salt tolerant and their mechanism of action involves interacting with and permeabilizing bacterial membranes and inhibition of macromolecular synthesis [30,32–34]. The ability of reproductive tract specific defensins and defensin-like proteins and peptides to display antimicrobial
activity against E. coli and reproductive tract pathogens projects them as potential therapeutic agents to treat sexually transmitted diseases. Current regimens to treat sexually transmitted diseases such as epididymitis involve the administration of antibiotics. For example, when Chlamydia trachomatis and N. gonorrhoeae are the cause of infection, ceftriaxone and doxycycline are used, whereas when coliform bacterial infections are suspected, ofloxacin or levofloxacin is recommended [35]. Development of resistance by pathogens to conventionally used antibiotics has led to the identification and characterization of a variety of natural and synthetic peptide antibiotics that have the potential to be used to effectively treat infections. However, studies that demonstrate the effectiveness of microbial killing when antibiotics are used in combination with the natural reproductive tract antimicrobial peptides are unknown. In this study, for the first time, we demonstrate the synergistic antibacterial ability of reproductive tract antimicrobial peptides in combination with the commonly used antibiotics to treat genital infections. Results of this study provide vital information for the development of novel strategies to treat sexually transmitted diseases that involve using antibiotics in combination with reproductive tract specific antimicrobial peptides.

Methods

Antibiotics and Peptide Synthesis

Antibiotics used in this study—carbenicillin, ampicillin, ciprofloxacin, kanamycin, chloramphenicol, tetracycline, doxycycline, gentamicin, streptomycin and rifampicin—were obtained commercially (Sigma Aldrich, St Louis, MO). HE2α and HE2β2 C-terminal peptides (a kind gift from Dr Susan H. Hall and Dr Frank S. French, Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC) were individually tested in combination with each of the antibiotics. The amino acid sequences of the peptides used are shown in Figure 1. They were synthesized at the Peptide Synthesis Facility, University of North Carolina, Chapel Hill by standard fluoren-9-ylmethoxycarbonyl (f-moc) solid phase procedures using Rainin symphony multiple peptide synthesizer (Rainin Instrument, Woburn, MA). The purified peptides eluted as single peaks upon reverse phase HPLC and were further demonstrated to have the correct molecular weight by MALDI-TOF mass spectrometry.

Fractional Inhibitory Concentration Assay for Synergy

The synergistic antibacterial killing activity of HE2 peptides in combination with antibiotics was carried out by checker board analyses as described earlier [36] using E. coli XL-1 blue (Stratagene, La Jolla, CA) as the target organism. Though the incidence of epididymitis is lower with E. coli when compared with other reproductive tract pathogens, due to constraints in maintaining and culture of pathogenic organisms, E. coli was chosen as the target organism in this study. Initial dose dependent bacterial killing activity of HE2 peptides and antibiotics were analyzed by adding increasing amounts to the microtiter plate wells along with the bacteria to determine the MIC. Control wells were also maintained with no peptide or antibiotic added to the bacteria. Bacterial growth was measured by reading the absorbance at A600 18 h after the addition of the peptide or antibiotic. The MIC is read as the minimal concentration necessary to inhibit growth by at least 90%, when compared to the no peptide or no antibiotic control well. To determine the fractional inhibitory concentrations (FICs), 50 µl of Luria-Bertani medium was added to each well in a 96 well microtiter plate followed by addition of 50 µl of antibiotic to the wells (A1 to A8) in the first row of the microtiter plate and double dilutions added from row 1 to row 7. Then the peptide was added to the wells (1A to 1H) of the first column and double dilutions added from column 1 to column 7. The concentration of each antibacterial agent added ranged between 4X MIC and 1/16X MIC. With these dilutions, row 8 and column 8 serve as antibiotic only treated and peptide only treated controls, respectively. The 64th well (H8) serves as no peptide or no antibiotic control. To each well, 10 µl of bacteria corresponding to 1 × 10^8 CFU/ml were added and incubated at 37 °C for 18 h. The FIC index (FICI) was calculated by the following formula:

\[
FICI = \frac{\text{FIC of peptide} + \text{FIC of antibiotic}}{\text{MIC of peptide} + \text{(antibiotic)} / \text{MIC of antibiotic}}
\]

where (peptide) is the concentration of the peptide in the microtiter well that is the lowest inhibitory concentration of the peptide in its column or row and (MIC of peptide) is the MIC of the peptide alone; (antibiotic) and (MIC of antibiotic) are defined in the same way. An FICI of < 0.5 indicates synergy, whereas it is considered additive when the index is > 0.5 and < 1.0. An FICI of > 1.0 indicates antagonism. Assays were performed independently three times and the average FICI calculated.

Results

The MICs of the peptides and the antibiotics used in this study were initially determined. The MICs of HE2α and HE2β2 peptides were found to be 17.2 ± 0.6 and 6.4 ± 0.2 µM, respectively (Table 1). The MICs of the different antibiotics used in this study are also given in Table 1.

Our previous studies demonstrated the potent antibacterial killing ability of HE2α and HE2β2 peptides [30–33]. In order to determine whether these two peptides can interact and display enhanced bacterial killing, a checker board analysis was carried out. The average FICI was found to be 0.7 ± 0.1 when HE2α and HE2β2 peptides were used in combination, suggesting an additive nature of interaction (Table 2).

Development of synthetic or natural peptide antibiotics to treat diseases caused by antibiotic resistant pathogens has recently become a major area of investigation. Further, treating antibiotic resistant pathogens with antibiotics in combination with antibacterial peptides is an emerging strategy. To determine whether epididymal antimicrobial peptides can exhibit improved bacterial growth inhibition when used in combination with
antibiotics, checkerboard analyses were performed using HE2α or HE2β peptide and the commonly used antibiotics against E. coli. The nature of the interaction between HE2α peptide and the antibiotics seem to be synergistic as indicated by the average FICI (Table 2). Interestingly, a combination of ciprofloxacin or doxycycline (the most commonly used antibiotics to treat epididymitis) and HE2α peptide exhibited the best growth inhibition, with an FICI of about 0.26 ± 0.01. HE2β peptide when used in combination with various antibiotics exhibited synergistic effect (Table 3). Similar to HE2α peptide, its synergistic effect was best when used in combination with ciprofloxacin or doxycycline. The average FICIs of HE2/β peptide in combination with various antibiotics (ranging from 0.38 to 0.1) seems to be much lower than that observed for HE2α peptide (0.36 to 0.2).

**Discussion**

Treatment of reproductive tract infections is a global challenge and current regimens involve the use of antibiotics. Prolonged use of antibiotics leads to the development of pathogen resistance, which necessitates the identification of a variety of peptide antibiotics that are promising in treating diseases caused by these antibiotic resistant pathogens. A strategy to circumvent the problem of the emergence of antibiotic resistant bacterial strains is to use new antimicrobial compounds and/or combination therapy. The combination therapy is generally used to increase the *in vivo* activity, to prevent the emergence of drug resistance and to broaden the antimicrobial spectrum. Recently, the increasing incidence of reproductive tract infections and the need to design novel therapeutic approaches to counteract them provided impetus to efforts to identify and characterize novel antimicrobial proteins and peptides of the reproductive tract. Earlier, we demonstrated that HE2 proteins and their C-terminal peptides exhibit salt tolerant and structure dependent antimicrobial activities utilizing mechanisms involving permeabilization of both outer and inner bacterial membranes [30] and inhibition of macromolecular synthesis [32]. Further, these peptides have been shown to exhibit antibacterial activity against reproductive pathogens, viz. *N. gonorrhoea* and *S. aureus* [31]. There were earlier studies on the combined use of antimicrobial, antifungal and antiviral peptides to inhibit microbial growth in combination with conventionally used antibiotics or drugs [37–39]. However, to our knowledge this is the first report on the nature of interaction and ability of reproductive tract antimicrobial proteins and peptides to kill bacteria in combination with conventionally used antibiotics. Results of this study demonstrate that a combination of the synthetic HE2α and HE2β/2 peptides exhibit an additive inhibitory effect on *E. coli* growth. Moreover, HE2α or HE2β/2 peptide in combination with an antibiotic acts synergistically to inhibit bacterial growth. These results suggest that HE2α and HE2β/2 peptides are potentially valuable for the treatment of reproductive tract infections in combination with antibiotics.

Cationic antimicrobial peptides can cross the outer membrane of Gram-negative bacteria by the self-promoted uptake pathway [40], which involves the high affinity binding of the peptide to surface lipopolysaccharide, resulting in the displacement of divalent cations that stabilize adjacent lipopolysaccharide molecules [41,42] leading to destabilization of the outer membrane. Our previous studies demonstrate that HE2 peptides bring about bacterial killing by membrane permeabilization and inhibition of macromolecular synthesis. It is possible that the synergistic effect observed in this study could be due to enhanced entry of antibi-otic into the bacterial cell through the membrane pores created by the peptide. Synergistic action between antimicrobial peptides and antibiotics that involves membrane permeabilization was previously shown for a variety of peptides such as the α helical peptide p18 [38], menstrual hemocidin [43] and defensins [44]. The nature of interaction between the defensins and...
antimicrobial proteins and peptides of the reproductive tract has been demonstrated earlier. For example, cathelicidins or the human CAP18/LL37 can act synergistically with defensins to bring about bacterial killing [45]. Though antimicrobial peptides that cause pores in the membrane are expected to increase the uptake of antibiotics when used in combination, this effect alone was found not to be sufficient to show synergistic effects. For example, synergy was not observed when synthetic peptides that have the ability to permeabilize the membranes of E. coli were used in combination with vancomycin or ampicillin [46], suggesting that increased access of intracellular targets to antibiotics due to membrane permeabilization by peptides as well as the secondary effects that the peptides can effect are important for synergy. The synergistic bacterial killing observed when HE2 peptides were used in combination with common antibiotics could be due to their ability to form pores in the membrane facilitating increased entry of antibiotics as well as the secondary effects of these peptides, i.e. inhibition of macromolecular synthesis.

In this study, we observed that a combination of HE2α and β2 peptides exhibited an additive effect. The inability of HE2α and β2 peptides to act synergistically with each other could be due to their similar mechanisms of action on a single target, the bacterial membrane. On the same lines, basing on previous studies it should be mentioned that synergy is not necessarily observed when antimicrobial peptides are used in combination with commonly used antibiotics. Absence of synergism has been attributed to various factors that govern the activity of lytic peptides. For example, no synergy was observed when synthetic antimicrobial peptides were used in combination with antibiotics against S. aureus [46]. Similar observation was made when bovine lactoferrin was used in combination with various antibiotics [47]. The absence of synergistic effects in these cases was due to the low MICs of the peptides used and it becomes experimentally difficult to assess synergy. On the same lines, it is also noteworthy to mention that depending on the chemical structures of antibiotics used in combination with polyethyleneimine, a polycationic synthetic polymer, the effects were either synergistic or antagonistic or indifferent [48]. PGLa, a synthetic antimicrobial peptide, exhibits synergy with magainin (containing a 23 amino acid hydrophobic tail) but not with certain synthetic peptides that lack this tail [46]. Varying structural features of lytic peptides may allow aggregation or competition between the peptides to bind to the membranes of target organisms, thereby making it difficult to measure the synergistic actions.

In conclusion, we report that the antibacterial peptides of the male reproductive tract exhibit synergistic bacterial killing when used in combination with the conventionally used antibiotics. Results of this study may provide vital information to develop novel strategies to treat reproductive infections.

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References

SYNERGISTIC ANTIMICROBIAL ACTIONS OF MALE REPRODUCTIVE TRACT PEPTIDES


Characterization of a Novel Lysozyme-Like 4 Gene in the Rat

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Abstract

Lysozyme-like proteins (LYZLs) belong to the class of c-type lysozymes and are not well characterized in many species including the rat. In this study, using in silico and molecular biology techniques, we report the identification, cloning and characterization of rat LYZL4 gene and also determine the expression pattern of LYZL1, LYZL3 and LYZL6. The rat LYZL genes were found to be distributed on three chromosomes and all of them retained the characteristic eight cysteine signature of c-type lysozyme. Homology modeling of rat LYZL4 indicated that its structure is similar to that of the mouse SLLP1. In the male reproductive tract of rat, LYZL gene expression was confined to the testis. LYZL1 and LYZL4 were found to be expressed in tissues beyond the male reproductive tract, whereas LYZL3 and LYZL6 were not. LYZL expression in the developing (10–60 day old) rats was androgen dependent in the testis. Immunodetection using antibodies against rat LYZL4 revealed the presence of LYZL4 protein in the germinal layer of the testes and on the sperm tail. Recombinant LYZL4 did not exhibit antibacterial, muramidase and isopeptidase activities characteristic to c-type lysozyme. To the best of our knowledge, for the first time we report the characterization of LYZL genes in the rat. Results of our study indicate that rat LYZL proteins may have an important role in male reproductive tract function.

Introduction

In the 1930s Alexander Flemming discovered lysozyme (EC 3.2.1.7), a remarkable bactericidal agent [1]. Basing on their physical and functional properties, a wide variety of lysozymes have been identified. They are mainly classified into six families, namely, g-type (goose type), c-type (chicken-type), phage, bacterial and plant [2]. Among them, the c-type are widely distributed across the species [3,4,5,6] and in various organ systems including the male reproductive tract. C-type lysozymes are N-acetylmuramidase binding proteins and are of two types, namely, the non-calcium binding c-lysozymes and the calcium-binding c-lysozymes [7]. The enzymatic action of c-type lysozyme involves the hydrolysis of beta-1,4 glycosidic bonds between C-1 of N-acetylmuramic acid and C-4 of N-acetylmuramic acid in the peptidoglycan of bacterial cell walls. Its ability to act between C-1 of N-acetylmuramic acid and C-4 of N-acetylglucosamine is characteristic to c-type lysozyme. To the best of our knowledge, for the first time we report the characterization of LYZL genes in the rat. Results of our study indicate that rat LYZL proteins may have an important role in male reproductive tract function.

In humans, besides the c-lysozyme, lysozyme like genes were identified [19] and some of them (LYZL2, LYZL/SLLP1, LYZL4 and LYZL6) are found to be expressed in the male reproductive tract [17,18]. Spermatozoa incubated with antibodies to human SLLP1 failed to fertilize eggs, thereby demonstrating a role in male reproductive function [18]. Similarly, incubation of spermatozoa with the mouse LYZL4 antibodies resulted in loss of fertilizing ability [20]. However, the role of other three mouse c-lysozymes in the reproductive tract is not yet clear. Unlike the human and mouse counter parts, the rat LYZL genes are not characterized. In the rat genome available at GenBank, of the four c-type lysozymes (LYZL1, LYZL3 and LYZL6), LYZL4 sequence is predicted, whereas the other three were annotated using the whole genome shot gun analyses. Except for their gene identification, the functional role is not reported till now.

In this study, we report the identification and characterization of the rat LYZL4. Further, the expression profile of the LYZL transcripts (LYZL1, LYZL3, LYZL4 and LYZL6) was analyzed and their androgen dependence determined. Their possible contribution to the male reproductive tract immunity was analyzed.

Results

In silico analyses

Using gene specific primers, rat LYZL4 mRNA transcript was amplified and sequenced. It is located on chromosome 8, whereas LYZL1, LYZL3 and LYZL6 are present on chromosome 10 and 17 (Figure 1). The LYZL4 sequence was submitted to GenBank and was
Figure 1. Rat Lyz gene localization. Arrows indicate direction of transcription. Positions were taken from the Mapview (RGSC v3.4) at the National Center for Biotechnology Information (NCBI) website.
doi:10.1371/journal.pone.0027659.g001
assigned the accession number HM125534. In silico protein translation analyses revealed that LYZL4 is encoded by four exons (Figure 2), which is in agreement with the predicted LyzL sequence available at GenBank. It is thought to be secretory because of the presence of a signal peptide. The predicted physical characteristics of the rat LYZL proteins are given in Table 1. An important feature is that all the rat LYZL proteins retained the characteristic eight cysteine signature of c-type lysozymes (Figure 3A). In LYZL4 one of the essential amino acids (aspartate) of c-type lysozyme active site is replaced by glycine (Figure 3A). Similarly, in LYZL3, aspartate is replaced by asparagine, suggesting that LYZL3 and LYZL4 may not exhibit lysozyme activity. However, the essential amino acids of the active site are conserved in LYZL1 and LYZL6 (Figure 3A). Loss of aspartate in the lysozyme active site was also observed in the human and mouse LYZL4 (Figure 3B). Sequence analyses reveal that the rat LYZL proteins are highly homologous to their mouse and human counterparts (Table 1). Similarly, based on the ClustalW2 score, the homology among the rat LYZL proteins was also found to be high (Table 2). Rat LYZL4 displays a high degree of homology with mouse SLIP1 (Figure 3C). Homology modeling using mouse SLIP1 as the template was carried out to determine the three dimensional structure of rat LYZL4 (Figure 4A and 4B). LYZL4 seems to be structurally similar to the mouse SLIP1 except at few residues as shown in the superimposed image (Figure 4C). There are 7 helices and 4 disulphide bridges that are conserved between...
the mouse SLLP1 and rat LYZL4. The beta sheets present between residues 43–60 in mouse counterpart are absent in the rat LYZL4. A change in secondary structure pattern was also observed near residue 23 wherein a helix is formed in case of rat LYZL4. A change in secondary structure pattern was also observed near residue 23 wherein a helix is formed in case of mouse SLLP1 and not in rat LYZL4. According to Ramachandran plot, 90.8% of the residues lie in the most favored regions and 9.2% in the additionally allowed regions (Figure 4D). There are no disallowed regions predicted. The generated model seems to be reliable with the good Ramachandran plot values with a G-factor of -0.12 (Figure 4D). The RMSD value was 0.405 which was within the agreeable limit. The similarity in structural and tissue localization of rat LYZL4 and mouse SLLP1 suggests that they may exhibit similar function.

**Lyz1 gene expression in the rat**

Using RT-PCR analyses, the expression pattern of rat *Lyz1* genes was determined in the male reproductive tract. All the *Lyz1* genes analyzed in this study were found to be expressed specifically in the testes (Figure 5). To determine if the expression pattern of *Lyz1* mRNA transcripts is male reproductive tract specific, RT-PCR was performed in a variety of tissues obtained from male and female rats. *Lyz1* was found to be expressed in the heart, lung and spleen, whereas *Lyz4* was found to be expressed in the brain, lung, ovary and uterus (Figure 6). *Lyz3* and *Lyz6* expression was not found in these tissues suggesting that their expression is highly male reproductive tract specific (Figure 6).

**Androgen dependent expression**

Gene expression in the male reproductive tract is under the influence of androgens [21,22]. To elucidate the influence of androgen variation, PCR analyses for *Lyz1* were carried out using total RNA isolated from the epididymides and testes of 10–60 day old rats. Though the expression of *Lyz1* transcripts is absent in the epididymis obtained from the adult rats (Figure 5), it is possible that they may be expressed in the younger rats during postnatal development. In the epididymis, none of the *Lyz1* analyzed in this study were expressed at all the ages during development (Figure 7A). In the testes, *Lyz1*, 3 and 6 were expressed starting from day 30 during postnatal development, whereas *Lyz4* was expressed in all the age groups (Figure 7B).

**Immunolocalization**

Since *Lyz4* was the only one predicted among the *Lyz1* analyzed in this study and that its expression was found to be present during all stages of postnatal development, we further studied its expression pattern at the protein level in the testis using polyclonal antibodies raised against LYZL4. It was found to be expressed in the germinal epithelium and concentrated on the developing spermatozoa (Figure 8). Using immunofluorescence, we observed that LYZL4 is expressed only in the tail region of the sperm obtained from adult rat (Figure 9), suggesting that LYZL4 may contribute to the motility of the spermatozoa.

**Muramidase, isopeptidase and antimicrobial activities**

LYZL4 being a c-type lysozyme is expected to exhibit the hydrolytic activity of glycosyl bonds. Hence, its muramidase and isopeptidase activities were analyzed. At the concentrations tested (1 and 5 μM) no activity was displayed by LYZL4 (Figure 10A and 10B). The positive control, lysozyme, displayed potent muramidase and isopeptidase activities. The antimicrobial activity of lysozyme is well known. To determine whether recombinant rat LYZL4 protein exhibits antimicrobial activity, its ability to kill *E. coli* was tested using colony forming unit (CFU) assay. LYZL4 (10–100 μg/ml) did not display any antibacterial activity at all the concentrations tested (Figure 10C). The negative control, LCN6, an epididymal lipocalin, did not show any detectable antibacterial activity when incubated for 2 h at concentrations up to 100 μg/ml (data not shown).

**Discussion**

C-type lysozyme is expressed in most species and because of its ability to act on microbial membranes, it is thought to play an important role in innate immune defense. In the recent years, lysozyme-like proteins were identified and characterized in the human and mouse [17,18,20]; their expression being reported in

**Table 1. General characteristic features of rat lysozyme-like proteins.**

<table>
<thead>
<tr>
<th></th>
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the male reproductive tract. The mRNA and protein expression pattern of human and mouse LYZLs varied within the male reproductive tract, suggesting that they may play different roles in these two species [17,18,20]. However, the expression pattern of lysozyme-like genes and proteins are not characterized in the rat. In this study, we analyzed their mRNA and LYZL4 protein expression to determine whether rat lysozyme-like gene function is similar to that of human and mouse.

In our rat genome mining, we identified four Lyzl genes distributed on chromosomes 8 (Lyzl4), 10 (Lyzl3 and 6) and 17 (Lyzl1). Basing on the accession number, Lyzl4 was found to be predicted. However, we did not find any literature that describes the identification and characterization of rat Lyzl genes. Hence, we submitted only the sequence of Lyzl4 to GenBank. In silico analyses revealed that Lyzl4 present on chromosome 8 is encoded by 4 exons, which is in agreement with the gene structure available at GenBank. Its homology with its human and mouse counter parts suggests that LYZL4 is highly conserved. The four rat Lyzl genes are distributed on three chromosomes. Such distribution of Lyzl genes on three chromosomes was also observed in human and mouse, indicating a possible organizational conservation. Homology between the rat LYZL proteins suggests that they might have originated from a common progenitor and may share a common physiological function. The presence of eight cysteine signature in the rat LYZLs supports the classification of these to the c-type lysozyme family.

Figure 3. Multiple sequence alignment of LYZL proteins. A) Rat LYZL proteins. B) Alignment of rat, mouse and human LYZL4 protein sequences. The conserved amino acid residues are shaded. Amino acids in the active site responsible for the enzyme activity are shown in red. The eight cysteines of the c-type lysozyme signature are indicated in bold and underlined. The LYZL4 sequence shown in bold was expressed as a recombinant protein. C) Alignment of rat LYZL4 and mouse SLLP1.

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Homology modeling reveals a close structural similarity of rat LYZL4 with mouse SLLP1. Such structural similarity suggests conservation of c-type lysozyme like proteins between the species.

To the best of our knowledge, we report for the first time the expression pattern of Lyz1 mRNA transcripts in the rat. Testis specific expression of Lyz1, 3 and 4 observed in this study implies a potential role for these genes in testicular function. Though Lyz1, 3 and 4 were expressed in other tissues, their expression was confined to the testis in the male reproductive tract. Such specific mRNA expression was previously reported for other Lyz1 genes. Lyz4 expression in the mouse was found to be testis specific [20]. Similarly, the human SLLP1 was found to be expressed specifically in the testis [18], whereas the other three human c-type lysozyme like genes identified were expressed in the testis/epididymides [17]. Reproductive tract specific expression was also demonstrated for other genes such as Spag11e [23], DEFB118 [24] and members of the HE2 family [13]. Contrary to the expression pattern of Lyz1 transcripts of human, the expression of rat Lyz1 genes in non-reproductive and female reproductive tissues suggests that they may have functions beyond male reproductive tract physiology. Similarly, the expression of Lyz1 and 3 mRNA transcripts in the mouse was also found to be present in other tissues beyond the reproductive tract. Results of this study and others reported earlier indicate that variability in tissue specific mRNA expression could contribute to the varied functional role of Lyz1 genes in different species.

Developmental regulation of a wide variety of genes due to the fluctuations of androgens at various stages in the male reproductive system has been studied extensively [25]. Androgen levels in the rat epididymis decline from birth until 20 days but remain at a substantial level of approximately 10 ng/g tissue (35 nM) until approximately 40 days when the levels begin to increase to that of the adult, between 15–20 ng/g [26]. Serum testosterone levels in
the young rat remain low and do not begin to increase to adult levels until 35–40 days of age [27]. Absence of Lyz1 transcripts in the epididymis obtained from 20–60 day old rats, suggests that their expression pattern is not androgen dependent in this organ system. Testicular androgen variation during development in the rat was reported to be significantly different from the epididymis. A steady increase in testosterone levels occurs in the rete testis of 30–130 day old rats [28,29]. In this study, the presence of Lyz1, 3 and 6 mRNA transcripts was observed in the testes starting from 30 day post natal development, whereas Lyz4 was expressed in all the age groups, though minimally during 10–30 days. The expression pattern of Lyz1 transcripts analysed in this study seem to correlate with the minimal androgen levels from day 20 to day 40 and increased androgen in the adult [26], suggesting that Lyz4 expression may be androgen dependent during development in the testis. Androgen dependent expression of Lyz4 during development was reported in the mouse [20]. Further studies are required to determine the molecular mechanisms that operate in controlling the expression of Lyz1 transcripts during development.

To demonstrate whether Lyz4 mRNA expression correlates with the protein expression, immunohistochemistry was performed on testicular sections. LYZL4 protein expression in the testes was observed in the germinal epithelium and on the maturing spermatzoa. It is possible that LYZL4 secreted into the lumen could bind to the sperm and aid in their development. Region specific gene expression of a wide variety of testicular and epididymal proteins on the sperm are reported [21]. The presence of LYZL4 specifically on the sperm tail suggests that it is involved in contributing to sperm motility. However, it is intriguing to note that though it is not expressed in the epididymis it is localized on the sperm tail. It is possible that LYZL4 is added on to the surface in the testis and this protein may continue to be present in the tail region in the epididymis.

The catalytic mechanism of c-type lysozymes involves the interaction of Glu-35 and Asp-52 of the active site with beta-1,4 glycosidic bond of the substrate. In this study, rat LYZL4 did not exhibit any muramidase and isopeptidase activity at the concentrations tested. This could be due to the replacement of aspartate by glycine in the catalytic site. Such loss of activity due to “changed” amino acids was reported for human SLLP1 and mouse LYZL4 [18,20] Epididymal proteins secreted into the lumen play a key role in sperm maturation. Besides this, some of them are known to exhibit potent antimicrobial activity, thereby forming important components of male reproductive tract innate immunity. Lysozyme, because of its ability to cleave the glycosyl bond of peptidoglycan, displays potent antimicrobial activity. In this study, we demonstrate that LYZL4 did not display any antibacterial activity against E. coli. The human c-type lysozyme like SLLP1, was non-bacteriolytic similar to the lack of antibacterial activity of rat LYZL4 observed in this study. The inability of rat LYZL4 to exhibit bacterial killing could be due to the modification in its active site.

In conclusion, for the first time, we report the identification of rat Lyz4 and the expression pattern of Lyz1, 3 and 6. In the male reproductive tract, their expression was confined to the testes. Lyz1 expression seems to be androgen dependent in the testes. Immunolocalisation revealed that Lyz1 mRNA is translated and the protein is localized on the germinal epithelium and on the sperm tail. LYZL4 did not exhibit antibacterial, muramidase and isopeptidase activities. Results of our study indicate that LYZL4 may play a crucial role in the testis and may also contribute to the motility of the sperm. Further studies are required to demonstrate the molecular mechanisms by which LYZL4 may contribute to these functions.

Materials and Methods

In silico analyses

Gene and protein notation used in this study was based on HUGO nomenclature. Gene symbols are italicised, with only the first letter in uppercase and the remaining letters in lowercase (Lyz1). Protein designations are the same as the gene symbol, but are not italicised, all uppercase letters (LYZL). The rat Lyz4 predicted sequence and Lyz1, Lyz3 and Lyz6 sequences were obtained from the rat genome (build RGSC v3.4) at the NCBI website (http://www.ncbi.nlm.nih.gov/). Gene specific
Primers were designed for each Lyzl mRNA (Table 3). RT-PCR was performed using rat testis mRNA as the template. The Lyzl4 PCR amplicons were sequenced, aligned and deposited in GenBank. The corresponding exon/intron boundaries were determined by aligning the cDNA with the genomic sequence. The sequences were translated and the predicted physical features of the deduced amino acid sequences were analyzed using tools available at ExPASy proteomics server (http://ca.expasy.org/).

The rat lysozyme like 4 (LYZL4) protein structure was predicted by homology modeling using MODELLER9v5. Basing on the BLAST search against PDB, the mouse sperm c-type lysozyme like protein 1 (SLLP1; PDB code 2GOI) was chosen as template because of its sequence similarity. The reliability of modeled structure was validated by Ramachandran plot analyses using PROCHECK and the correlation in structure between the template and model was verified by analyzing the RMSD values using PyMOL.

Tissue specimens and RT-PCR

Wistar rats (aged 60–90 days; n = 3) were obtained from National Institute of Nutrition, Hyderabad, India. Tissues collected were placed in RNA Later (Ambion Inc, Austin, TX, USA) solution overnight at 4 °C to allow penetration and fixation and stored at -70 °C. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the following tissues: the three regions of the epididymis (caput, corpus and cauda), testis, prostate, seminal vesicle, brain, liver, lung, kidney, heart, spleen, cervix, ovary and uterus. Total RNA (2 μg) was reverse transcribed using 200 U SuperScriptIII (Invitrogen, Carlsbad, CA, USA) and 0.5 μg of oligodT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. 2 μl of the resultant cDNA was amplified by PCR using gene specific primers (Table 1) for Lyzl1, Lyzl3, Lyzl4, Lyzl6 and Gapdh. PCR was performed under the following conditions: 94 °C for 2 min followed by 25–35 cycles at 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec, and with a final round of extension at 72 °C for 10 min. PCR amplicons were analyzed by electrophoresis on 2% agarose gels. For studies on the developmental regulation of Lyzl genes, epididymides and testes were collected from 10–60 day old Wistar rats (n = 3) purchased from the National Institute of Nutrition, Hyderabad, India.

Recombinant protein production

Recombinant LYZL4 protein was prepared as described earlier [30]. Briefly, the open reading frame that corresponds to the rat LYZL4 full length without the signal peptide (amino acid sequence shown in bold in Figure 3) was cloned into pQE30 expression vector (Qiagen, Valencia, CA, USA). E. coli (BL-21) was transformed with pQE30 vector containing rat Lyzl4 cDNA according to the supplier’s instructions. Fusion protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactoside for 3 h at 37 °C. 1% glucose was maintained in the medium to avoid baseline expression of the protein prior to induction. Bacterial lysate incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) for 1 h to allow binding of His-tagged recombinant protein to the resin, was then transferred to a
column, washed and eluted according to the manufacturer’s recommendations. The His-tagged recombinant LYZL4 protein contained the following additional amino acid residues at the N-terminus (MRGSHHHHHHGS) due to the construction of the vector. Fractions were analyzed on 15% gradient polyacrylamide Tris-Tricine gels and stained with Coomassie blue G250. Further, the identity of the protein was confirmed by Western blotting using anti-His-tag antibody. Fractions containing purified protein were pooled and dialyzed against phosphate buffered saline (pH 7.4) to remove urea.

**Antibody production and immunodetection**

Antibodies to detect rat LYZL4 were raised in our laboratory. Briefly, rabbits were immunized with recombinant LYZL4 protein mixed with complete adjuvant followed by booster doses 4 and 6 weeks after initial immunization. Antiserum was collected 2 weeks after the second booster dose. For immunohistochemical staining, testes were fixed in Bouin’s fluid and embedded in paraffin. Five micron thick sections were taken and treated with xylene and graded alcohol (70-100%). The sections were then treated with 1% Triton-X 100 to facilitate permeabilisation followed by treatment with 3% H2O2. LYZL4 was detected by incubating the sections using polyclonal antibodies (1:250 dilution) raised in rabbit followed by biotin conjugated secondary antibody (1:500 dilution) against rabbit IgG raised in goat. Immunostaining was detected using a Vectastain Elite ABC kit (avidin- biotin-complex horse radish peroxidase) (Vector Laboratories Inc., Burlingame, USA). Diaminobenzidine, the chromogen, produced a brown reaction product. Sections were counter-stained with hematoxylin. For the control staining, antibodies were preincubated with antigen (LYZL4 recombinant protein). Immunofluorescence on the sperm was detected by using anti-rabbit secondary antibodies tagged with FITC. Photographs were taken using a color digital imaging system attached to a Leica Photomicroscope. Surgical procedures

**Table 3. Gene specific primers used in this study.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz1</td>
<td>Forward</td>
<td>5’-TGTCGG TGT CTC CGC CCT AAT T-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GAC GAG TCT TCT CTA CAG T-3’</td>
</tr>
<tr>
<td>Lyz3</td>
<td>Forward</td>
<td>5’-TGCTG TGG CAG TCT TCT CCA TCC ACC A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TAG AAG TCA CAG CCA ACC ACC C-3’</td>
</tr>
<tr>
<td>Lyz4</td>
<td>Forward 1</td>
<td>5’-ATG TGG GCA CTA CGC ACC TTA A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 1</td>
<td>5’-CTG AGG ATT GAG GAT TCC TCC C-3’</td>
</tr>
<tr>
<td></td>
<td>Forward 2</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
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<tr>
<td></td>
<td>Reverse 2</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
</tr>
<tr>
<td></td>
<td>Forward 3</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 3</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
</tr>
<tr>
<td>Lyz6</td>
<td>Forward</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ATG TGG GCA CCA ATC GGA GTA TCC A-3’</td>
</tr>
</tbody>
</table>

Figure 10. Muramidase, isopeptidase and antibacterial activities of rat LYZL4. A, B) 1 (▲) and 5 (●) μM rat recombinant LYZL4 protein was incubated at 37°C and the formation of product due to muramidase and isopeptidase activities was measured spectrophotometrically at 450 and 405 nm respectively. 1.71 μM (25 μg/ml) lysozyme ( ■) was used as a positive control. C) Mid-log phase E. coli were incubated with 0 ( ■), 10 ( ▼), 25 ( ▲), 50 (●) and 100 ( ●) μg/ml rat recombinant LYZL4 protein for 0–120 min. Values shown are Mean ± S.D.

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were conducted using the guidelines for the care and use of laboratory animals and this study was specifically approved by the Institutional Animal Ethics Committee of University of Hyderabad (LS/IAEC/YS/11/07).

Antibacterial assay

Colonies forming units (CFU) assay was employed to test the antibacterial activity as described previously [30]. Briefly, overnight cultures of *E. coli* XL-1 Blue (Stratagene, La Jolla, CA, USA) were allowed to grow to mid-log phase (A600 = 0.4 – 0.5) and diluted with 10 mM sodium phosphate buffer (pH 7.4). Approximately 2×10^8 CFU/ml of bacteria were incubated at 37°C with 10–100 μg/ml of the LYZL4 recombinant protein and aliquots of the assay mix were taken at 30, 60, 90 and 120 min after the start of incubation. After incubation, the assay mixes were serially diluted with 10 mM sodium phosphate buffer (pH 7.4) and 100 μl of each was spread on a Luria–Bertani agar plate and incubated at 37°C overnight to allow full colony development. The resulting colonies were hand counted and plotted as log CFU/ml. As a negative control, the epididymal secretions of androgen-regulated epididymal sperm-binding protein from human. Antibacterial activity of antileukoprotease. Infect Immun 64: 4520–4524.


