Expression of a Novel Sphingosine 1-Phosphate Receptor Motif Protein Gene in Maturing Rat Testes

Ahmed Magzoub Khalid,1 Atsushi Asano,1 Yoshinao Z. Hosaka,2 Kenji Ohyama,3 Masanori Ohta,3 and Yoshiaki Yamano1,4

1Laboratory of Veterinary Biochemistry, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan
2Laboratory of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan
3Interdisciplinary Graduate School of Medical and Engineering Sciences, University of Yamanashi, Yamanashi 409-3898, Japan

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We screened a novel sphingosine 1-phosphate receptor type 5 motif-containing gene, LOC290876, from maturing rat testes by differential display. Gene expression was testis-specific, increased at week 7, and continued for 15 weeks. PCR analysis clarified two gene transcript isoforms, which were expressed at the same level in all samples detected in Northern blot. The deduced amino acid sequences of the two isoforms revealed differences in carboxyl terminal sequences. Gene and protein expression in the testes was dominant in the spermatocytes, and protein expression was localized to the nucleus. Taken together, these findings suggest that the LOC290876-encoded gene product is not involved in sphingosine signaling, but has distinct roles in the nucleus during the processes of spermatocyte maturation and meiosis producing spermatids.

Key words: sphingosine 1-phosphate receptor type 5 (EDG-8); immunohistochemistry; in situ hybridization; spermatogenesis; splicing variant

Spermatogenesis is a complex, programmed process, that involves the ordered expression of many genes within the testes. In the seminiferous tubules, spermatogonia stem cells attach to the basal membrane, divide by mitosis, and undergo meiosis producing spermatids from spermatocytes. This is known as the cycle of the seminiferous epithelium. More than 15,000 full-length mammalian cDNA sequences, or expressed sequence tags (EST), have been isolated, and using this information, spermatogenesis-related genes have been identified and determined. The DNA microarray is a powerful tool for screening these genes using isolated spermatogenic cell-derived mRNA as probes after separation by flow cytometry. Based on this technique, many spermatogenesis candidate genes have been isolated and analyzed. While the functions of some of these have been determined by comparing DNA sequences in the database, many functions remain unknown. Understanding expression specificity and gene profiling during spermatogenic processes in the testes should help to elucidate the biological functions of these gene products, which can be used as genetic markers to diagnose human infertility.

Recently, Yang et al. described the expression of novel gene GON-SJTU1 in rat testes, which was detected by 15 postnatal days, and then dramatically decreased after sexual maturation. Although the function of this gene is not well understood, information on the gene should be useful in diagnosing diseases such as cancer and infertility, as well as in understanding the molecular mechanism of spermatogenesis. We have screened, identified, and characterized many specific genes from maturing rat testes by differential display (DD). In the present study, we focused on candidate gene LOC290876, of unknown biological function. We analyzed its expression in maturing testes, and elucidated its expected function in spermatogenesis.

Materials and Methods

Animals, RT-PCR, Northern blotting, and in situ hybridization. Sprague-Dawley (SD) rats were purchased from CLEA Japan (Tokyo). Total RNA from testes at various developmental stages and from individual organs of 9-week-old male and female rats was prepared using TRIzol reagent (Life Technologies, Carlsbad, CA), following the manufacturer’s recommendations. DD was performed using 3-week- and 7-week-old rat testis RNA as template. Candidate cDNAs, showing higher expression at week 7 than at week 3, were cloned and sequenced, and their nucleotide sequences were compared with those in the National Center for Biotechnology Information (NCBI) DNA database. We chose one candidate, sphingosine 1-phosphate receptor motif protein gene LOC290876, accession no. NM_001037182. Gene expression was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting. RT-PCR primer sequences were designed as follows: upstream primer 5’-CCCATGACACAGGGTACTTGTCTG-3’, located in exon 6, and downstream primer 5’-GAAACCAGTTCTGGGAGCTTGT-3’, located in exon 9. cDNA was synthesized from 1μg of RNA using a first-strand cDNA synthesis kit, including Super Script III Reverse Transcriptase (Life Technologies). PCR was done using Takara Ex Taq DNA polymerase (Takara Bio, Shiga, Japan), according to the following schedule: 94°C for 5 min, and then 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, for 30 cycles, and finally 72°C for 10 min. For Northern blotting, 5μg of total SD rat RNA was electrophoresed on a formaldehyde-containing agarose gel and transferred to a Hybond N+.
Lysis-M (Roche). Proteins (30 μg of each sample) were electro-phoresed on a 10% polyacrylamide gel containing sodium dodecyl sulphate, which was transferred to a Hybond C membrane (GE Healthcare, Buckinghamshire, UK). LOC290876 cDNA was labeled with a random primer labeling system (GE Healthcare) using [α-32P]dCTP, hybridized, and then rehybridized with rodent Gapdh cDNA as internal standard.

LOC290876 expression in the testis section was analyzed by in situ hybridization (ISH), as described previously.11) In brief, DIG-labeled sense and antisense riboprobes were hybridized with a 5-μm section of the testis of an 8-week-old rat. The probe was designed in the area used for RT-PCR amplification. Signals were detected using a DIG Nucleic Acid Detection Kit (Roche Applied Science, Tokyo).

Western blotting and immunohistochemistry. Protein expression was measured by Western blotting. Two synthetic oligo-peptides, C + ASEKNIHQPSQDGTPPL and CIRKLLRKELDSEDQS, were used to immunize rabbits, and sera were collected as polyclonal antibodies by a custom antibody producing service (Operon Biotechnologies, Tokyo). Rat tissue proteins were prepared and purified using Complete by a custom antibody producing service (Operon Biotechnologies, Tokyo). Protein expression in the testes was analyzed by immunohistochemistry. Protein expression in the testis section was analyzed by Western blotting and immunohistochemistry. Another testis section used in ISH was hybridized with the primary antisera (1:500) used for Western blotting, and signals were visualized using the Histofine DAB Substrate kit (Nichirei, Tokyo) following the manufacturer’s protocol.

Results

RT-PCR and Northern blotting
LOC290876 gene expression was analyzed by RT-PCR and Northern blotting. The mRNA size of the gene was 1,454 bp, and the Northern blotting result confirmed this gene size. Two strong bands of similar molecular size were detected in the 7-week to 15-week-old rat testes, and expression in the 9-week-old rats was testis-specific. The band sizes were slightly smaller than those of 18S rRNA (Fig. 1A), and suggest the presence of splicing variants. To clarify this, we designed PCR primer combinations, that covered the entire cDNA area. PCR amplification using primers in exons 6 and 9 produced two bands at 489 bp and 373 bp (Fig. 1B). No multiple amplifications were observed using other primer combinations (data not shown). We determined the sequences of the two fragments, which we named LOC290876-FL (full length isomer) and LOC290876-TL (truncated isomer). LOC290876-TL contained a 116-nucleotide deletion at the 5′ boundary of exon 8 (Fig. 2). Part of the LOC290876-TL isomer sequence is to appear in the DNA Data Bank of Japan (DDBJ) under accession no. AB714639. The two isomers encoded a common sequence of 216 amino acids at the amino termini, while the carboxyl termini showed differences in amino acid length (21 versus 24 amino acids). The LOC290876-TL-encoded carboxyl terminal 24 amino acid sequence, deduced from the DNA sequence, had no homology with the other proteins.

Protein localization
The Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences (PSORT) program (http://psort.hgc.jp/) is commonly used to as to the presence of the signal peptide and subcellular localization of novel proteins by applying the predicted amino acid sequences.12) By this analysis, we found no signal peptide in the protein, suggesting that the encoded protein is not secreted. Instead, it is possible that it is a soluble protein localized in the nucleus.
Western blotting

The deduced coded protein of LOC290876-FL was determined to be 237 amino acids long. It was an acidic protein (pI 4.8) with three sphingosine 1-phosphate receptor type 5 (EDG-8) motifs according to the Ensembl survey (www.ensembl.org). Protein expression at various developmental stages of the testes and in various organs of the 9-week-old rats was determined by Western blotting. Two bands of almost the same size (26.6 kDa and 26.2 kDa) and intensity were increased at the 7-week-old rat testes. Expression was testis-specific and remained at almost same level until 15 weeks (Figs. 1C and 2).

In situ hybridization and immunohistochemistry

LOC290876 mRNA expression was analyzed by in situ hybridization in seminiferous tubule sections according to the seminiferous epithelial cycles (Fig. 3). LOC290876 mRNA expression was widespread in the cytoplasm of the spermatocytes in the individual tubules. Protein expression of the gene product was determined by immunohistochemistry and was observed mostly in the nuclei of spermatocytes (Fig. 4). Both LOC290876 mRNA and protein expression were observed at specific stages of spermatocyte development, as indicated by arrows in the figures.

Discussion

To elucidate individual biological processes and the physiological functions of organisms, it is necessary to collect information about cell-specific genes and to analyze their expression. In the case of spermatogenesis, many genes have been analyzed by RT-PCR and DNA microarray, but not all of their functions have been determined. Nevertheless, gene collection represents a tool for the diagnosis of azoospermia and of specific cancers, several cancer-testis (C/T) antigens are expressed in these cancer cells, and expression of them is restricted to developing testes in normal organs.11)

We are interested in elucidating the biological functions of these so-called orphan genes, including that of LOC290876, which was found in the present study to contain three EDG-8 motifs according to the Ensembl survey. Ten specific elements have been identified in the EDG-8 sequence, and three of them are located in LOC290876 product sequences. These three domains have related sequences in EDG-8: e.g., lies in the N-terminus; 2 spans the second cytoplasmic loop and leads into transmembrane domain; and 3 lies in the third cytoplasmic loop. The mRNA size of LOC290876 has been reported to be 1,454 bp, in agreement with the results of Northern blotting in this study. Rat sphingosine 1-phosphate receptor type 5 cDNA size was reported to be 2,171 bp (accession no. NM_021775). Sphingosine 1-phosphate is known to be involved in several biological processes, including mitosis and differentiation, and may be involved in cancer and angiogenesis.13) Sphingosine 1-phosphate receptors are G-protein coupled membrane proteins and these proteins are divided into subtypes that show organ-specific expression.14) For example, higher expression of EDG-8 occurs in the adult rat brain and spleen as compared with low expression in the testes.15) Since no expression of LOC290876 mRNA was detected in the adult rat brain and expression was localized to the nucleus, according to the results of immunohistochemistry, we concluded that this gene product has a function different from EDG-8.

To determine protein structure and subcellular localization, we carried out PSORT analysis. The protein was
predicted to be soluble and localized in the nucleus, in good agreement with our immunohistochemical observations. Recently, Schultz-Thater et al. described a novel C/T antigen gene, MAGE-A10, that encodes a protein expressed in the nuclei of spermatogonia and spermatocytes.16) Such expression and localization characteristics are similar to the product encoded by LOC290876, suggesting that these proteins have similar functions, for example, as nuclear gene expression regulators.
Finally, we identified two splicing variants of LOC290876. In the truncated isoform, exon 7 was joined to the AG splicing acceptor in exon 8 resulting in truncated mRNA. The proteins encoded by the two isoforms differed only in their amino acid sequences at the carboxy-terminal, and the LOC290876-TL-encoded carboxyl terminal showed no homology with the other proteins. There are many reports of splicing variants in spermatogenesis, which appear to vary in biological function and regulation. For example, two variants have been reported in the testicular zinc finger protein (TZF) and in LIM domain-containing protein genes, which show increased expression in maturing testes. Since the two LOC290876 isoforms share long common amino acid sequences in their amino termini and similar expression profiles, it is possible that they have both common and distinct functions in spermatogenesis, especially during the process of meiosis, since expression of them was so widespread in spermatocytes. This result is in agreement with the result of DD, in which mRNA expression was upregulated after 7 weeks in the rat testes when pachytenes spermatocytes were observed.

These findings suggest that the LOC290876-encoded gene products are not involved in sphingosine signaling, but rather have distinct roles in the nucleus during the process of spermatocyte maturation and meiosis producing spermatids. The gene knockdown experiment is a useful procedure to understand the physiological function of a novel gene product, and the Cre-LoxP system is a useful procedure to understand the physiological function and regulation. For example, two variants have been reported in the testicular zinc finger protein (TZF) and in LIM domain-containing protein genes, which show increased expression in maturing testes.

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