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Molecular characterization of a germ-cell-specific antigen, TEX101, from mouse testis

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Summary

TEX101, a glycoprotein we recently identified, is primarily characterized as a unique germ-cell-specific marker protein that shows sexually dimorphic expression during mouse gonad development. Based on data obtained from molecular biological as well as immuno-morphological studies, we believe this molecule may play a role in the process underlying germ cell formation. However, many points remain unclear as the molecular characteristics and its physiological functions are far from being completely understood. To clarify the molecular basis of TEX101, we herein report a further biochemical characterization of the molecule using testicular Triton X-100 extracts from mice. Deglycosylation studies using endoglycohydrolases that delete *N*-linked oligosaccharides (OS) from the molecule show that TEX101 is highly (approximately 47%) *N*-glycosylated. All potential *N*-glycosylation sites within TEX101 are glycosylated and most of these sites are occupied by endoglycosidase F2-sensitive biantennary complex type OS units. In addition, an extremely low population among TEX101 possesses only endoglycosidase H-sensitive hybrid type OS units. In studies using phosphatidylinositol-specific phospholipase C against native testicular cells or *TEX101* transfectant, the enzyme treatment caused major reduction of the TEX101 expression on the cell, suggesting that TEX101, at least in part, is expressed as a glycosylphosphatidylinositol-anchored protein. Taken together, these findings will help elucidate the molecular nature of TEX101, a marker molecule that appeared on germ cells during gametogenesis.

Keywords: GPI-anchored protein, Glycoprotein, Spermatogenesis, Testis, TEX101

Introduction

Our previous studies identified and provided an initially characterization of a stage-specific marker molecule of germ cells, TEX101, in mice (Kurita *et al.*, 2001; Takayama *et al.*, 2005*a*, *b*). Using a monoclonal antibody (mAb) against mouse testicular homogenate, TEX101 was originally identified as a novel molecule specifically expressed on germ cells in the adult mouse testis (Kurita *et al.*, 2001). Immunoelectron microscopic studies showed that the molecule was predominantly located on the plasma membrane of spermatocytes and spermatids (Kurita *et al.*, 2001). Further immuno-morphological studies demonstrated that TEX101 was present on germ cells in both male and female gonads during pregametogenesis (Takayama *et al.*, 2005*a*). In the testis, TEX101 was expressed constitutively on prespermatogonia before initiation of spermatogenesis (Takayama *et al.*, 2005*a*). The molecule then interestingly disappeared from the surface of germ cells when the prespermatogonia differentiated into spermatogonia, but reappeared on spermatocytes and spermatids (Takayama *et al.*, 2005*a*). In the ovary, TEX101 was primarily detected on germ

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cells from around 14 days post-coitus until the start of folliculogenesis in the developing ovary, but was not detected on oocytes surrounded by follicular cells (Takayama *et al.*, 2005*a*). Indeed, the initial study by Northern blot analysis demonstrated that the *TEX101* message was not detectable in adult mice ovaries (Kurita *et al.*, 2001); reverse transcription (RT)-polymerase chain reaction (PCR) analysis of *TEX101* further showed that the message of the *TEX101* transcript was faintly detected at 4 days post-partum (dpp), and no longer detectable after 6 dpp in the ovary (Takayama *et al.*, 2005*a*). In addition, although TEX101 remained on the cell surface of spermatids and testicular sperm, the protein was shed from epididymal sperm in the caput epididymis (Takayama *et al.*, 2005*b*).

Molecular biological analysis of TEX101 revealed that the coding region of the TEX101 cDNA (GenBank accession no. AB022914) contains 750 base pairs (bp), translating to 250 amino acids (Kurita *et al.*, 2001). Sequence analysis of the cDNA clone revealed no homologous molecule in the DNA/protein database, suggesting that the molecule may have a unique role in the process of gametogenesis. After removal of the putative 25 amino acid signal peptide at the N-terminus, the molecular weight of the TEX101 mature protein (225 amino acids) was found to be 24 093 as calculated from the predicted amino acid sequence. While the TEX101 protein had an apparent molecular mass of 38 kDa under the non-reducing conditions analysed by Western blotting (Kurita *et al.*, 2001; Takayama *et al.*, 2005*b*), this relatively large difference in the molecular mass may be attributable to glycosylation of the peptide, because four potential *N*-glycosylation sites and several possible *O*-glycosylation sites (Ser/Thr residues) were found in the deduced amino acid sequence of TEX101 (Kurita *et al.*, 2001). According to experimental results on the subcellular localization of TEX101 within the testis, the TEX101 protein had a hydrophilic form that was detected in the testicular water-soluble fraction (Kurita *et al.*, 2001). However, the majority of TEX101 was found in the testicular Triton X-100-soluble fraction (Kurita *et al.*, 2001), suggesting that the protein may strongly associate with the membrane portion of the testicular cells after biosynthesis. These data also suggest that the TEX101 protein may have two forms, i.e. a cytosolic form and a glycosylphosphatidylinositol (GPI)-anchored form, since the hydropathy plot analysis revealed two putative signal sequences at both the N- and C-terminals of the molecule, a typical characteristic of GPI-anchored proteins (Yeh *et al.*, 1994; Udenfriend & Kodukula, 1995; Kinoshita *et al.*, 1997). Although the rat homologue of TEX101 (TEC-21) was reported as a GPI-anchored protein (Halova *et al.*, 2002), little or no information is available on the molecular nature of TEX101.

To determine the molecular importance of TEX101, we are currently undertaking further characterization of the molecule, including promoter analysis (Tsukamoto *et al.*, 2006). Here, we report the molecular characterization of TEX101 using endoglycohydrolases and TEX101 transfectant to more precisely understand its molecular nature.

Materials and methods

Animals and chemicals

Sexually mature male ICR or BALB/C mice (8–10 weeks old) were purchased from Charles River Japan (Yokohama, Japan). They were maintained under standard conditions (12L:12D) and given free access to water. All animal experiments were conducted according to the guide for care and use of laboratory animals, Juntendo University.

Recombinant *N*-glycanase and endoglycosidase H (endo-H) were from ProZyme Incorporation (San Leandro, CA). Endoglycosidase F2 (endo-F2; purified from *Flavobacterium meningosepticum*) was from Genzyme Corporation (Cambridge, MA). Phosphatidylinositol-specific phospholipase C (PIPLC) was from either Sigma-Aldrich (St Louis, MO), or Molecular Probes (Eugene, OR). Restriction enzymes were obtained from Roche Diagnostics (Penzberg, Germany). TaKaRa Ex HS polymerase and T4 DNA ligase were from TaKaRa (Shiga, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from American Type Culture Collection (Manassas, VA). A mouse mAb to TEX101 (TES101: mouse IgG1) was produced and purified as previously described (Kurita *et al.*, 2001). A mAb, 3H9 (mouse IgG1), specific for GPI-80 (Ohtake *et al.*, 1997; Suzuki *et al.*, 1999), a GPI-anchored protein mainly identified on human neutrophils (Suzuki *et al.*, 1999), was used for control studies. Fluorescein isothiocyanate (FITC)-, or horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (Ig) antibody $F(ab')_2$ fragment was purchased from DAKO (Glostrup, Denmark). Penicillin G, streptomycin, pCRII-TOPO vector, pcDNA3.1(−) vector and lipofectamine 2000 transfection reagent were from Invitrogen (Carlsbad, CA). BigDye terminator v3.1 cycle sequencing kit was from Applied Biosystems (Foster City, CA). All other chemicals were obtained commercially and were of highest purity available.

Cells

HEK293 cells, human embryonic kidney cell line (Graham *et al.*, 1977), were obtained from the American Type Culture Collection.

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Preparation of cellular extracts and Western blot analysis

Mouse testicular extract using Triton X-100 was essentially prepared according to the method described previously (Kurita *et al.*, 2001). Cellular extract of HEK293 transfectant (see below) was prepared as follows: Approximately 2×10^5 cells were suspended in 50 µl of 10 mM Tris-HCl (pH 7.5)/150 mM NaCl containing 1× concentration of Complete (Roche) and 30 mM *n*-octyl-β-D-glucoside (Wako Pure Chemicals Industries, Osaka, Japan) with gentle pipetting and incubated on ice for 15 min. After incubation, the sample solution was centrifuged at 18 000 *g* for 15 min at 4 °C. The supernatant was used as HEK293 cellular extract for further analysis. Protein concentration of the cellular extracts was determined by the Bio-Rad microprotein assay using bovine serum albumin as a standard. The protein solutions were separated by the SDS-PAGE system (Laemmli, 1970) under non-reducing conditions. Some samples were deglycosylated with either *N*-glycanase, endo-H or endo-F2 before electrophoresis according to the method described previously (Araki *et al.*, 1992*a*; Anzai-Takeda *et al.*, 2005). After blotting to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) from the SDS-PAGE gel (Araki *et al.*, 1992*b*; Kurita *et al.*, 2001), the reactivity of the transferred protein(s) with TES101 antibody (0.6 µg/ml) was assayed using HRP-conjugated secondary antibody and visualized using an ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Yu *et al.*, 2000; Takayama *et al.*, 2005*b*).

Construction of TEX101 transfectant

TEX101 expression vector was prepared as follows: An EST514549 clone (80 pg) containing mouse TEX101 ORF (Research Genetics, Huntsville, AL) was amplified by PCR using forward *Bam*HI-TEX101-F primer (5′-GGATCCATGGGAGCCTGCCGCATC-3′: *Bam*HI sensitive sequence is underlined, 0.5 µM) and reverse *Hin*dIII-TEX101-R primer (5′-AAGCTTCTTCAAGGGAAGTGGGTG-3′: *Hin*dIII sensitive sequence is underlined, 0.5 µM) in a 30 µl of 1× Ex Taq buffer containing dNTP mixture (0.2 mM each) and TaKaRa Ex HS polymerase (0.05 unit/μl). Thermal cycling conditions consisted of 1 cycle of 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s, and 1 cycle of 72 °C for 7 min. The PCR product was subcloned into a pCRII-TOPO vector. The insert containing TEX101 ORF was excised by *Bam*HI and *Hin*dIII double digestion, then subcloned into a pcDNA3.1(−) vector to be as pCD-TEX101. The oligonucleotide sequences were confirmed by a BigDye terminator v3.1 cycle sequencing kit using Genetic Analyzer 3100 (Applied Biosystems). HEK293 cells were maintained in DMEM supplemented with 10% of heat-inactivated FCS, 100 unit/ml penicillin G and 100 µg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. The cells were seeded at a density of 5×10^5 cells per 60 cm^2 culture dish in 4 ml of growth medium and cultured for 24 h. Subsequently, they were transfected with either pCD-TEX101 (8 µg) or pcDNA3.1(−) vector (8 µg) using 20 µl of lipofectamine 2000 transfection reagent, respectively. After 36 h of transfection, they were used for further experiments.

PIPLC treatment and immunohistochemistry

PIPLC-treatment was carried out as described previously (Sayama *et al.*, 1991). Briefly, freshly prepared, unfixed testes from adult BALB/c mice were embedded in Tissue-Tek OCT compound (Sakura Finetechnical. Tokyo, Japan) and flash-frozen in liquid nitrogen. Serial tissue sections (5 µm thick) were made with a Microm HM 550 cryostat (Microm, Walldorf, Germany), mounted on round glass coverslips (13 mm diameter, No. 1 thickness; Matsunami, Osaka, Japan), coated with 2% 3-aminopropyltriethoxy-silane (Sigma-Aldrich), and then allowed to air dry. The samples were then fixed in acetone for 10 min at 4 °C. After washing with phosphate-buffered saline (PBS), the samples were incubated with PBS containing 0–2.0 U/ml of PIPLC at 4 °C for 30 min. The remaining TEX101 on the sections was immunostained with TES101 mAb (0.26 µg/ml) followed by incubation with Alexa Fluor 594-labelled goat anti-mouse IgG (10 µg/ml) (Molecular Probes). Fluorescence and differential interference contrast (DIC) images were collected with a BX60 microscope (Olympus, Tokyo, Japan) equipped with a Spot RT SE6 CCD camera (Diagnostic Instruments, Sterling Heights, MI) and captured with the MetaMorph image analysis system (Universal Imaging, Dowingtown, PA). The relative levels of TEX101 present in the PIPLC-treated sections were determined by measuring the fluorescence intensity.

PIPLC treatment and flow cytometry

The TEX101 transfectant (5×10^5,/ml) was incubated in PBS (pH 7.4) containing 1 U/ml PIPLC for 45 min at 37 °C, then the cells were washed with PBS twice. After washing, the cells were treated with TES101 or isotype-matched control antibody (3H9; concentration 3 µg/ml) for 30 min at 4 °C. Then the cells were treated with 5 µg/ml FITC-conjugated rabbit anti-mouse Ig antibody for 30 min at 4 °C, and washed twice with PBS. The expression of TEX101 on the cell surfaces was measured by a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Dead cells and debris were excluded from the analysis by forward and side scatter gating.

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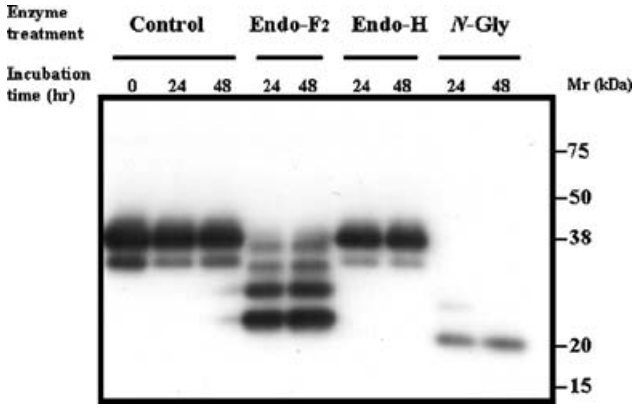

Figure 1 Western blot analysis of the testicular Triton X-100 fraction probed with anti-TEX101 mAb (TES101) after endoglycosidase digestion. Aliquots containing 50–100 µg of testicular Triton X-100 extract were digested with either endoglycosidase F2 (Endo-F2: 10 mU/ml), endoglycosidase H (Endo-H:50 mU/ml) or *N*-glycanase (*N*-Gly: 100 mU/ml) in a total volume of 30–100 µl as described elsewhere (Araki *et al.*, 1992a; Anzai-Takeda *et al.*, 2005). The reaction mixture was incubated for either 24 h or 48 h at 37 °C. For the 48 h treatment, additional enzymes (half of the original amount) were added to each tube 24 h after the start of the incubation, and the mixture incubated for an additional 24 h at 37 °C. After the incubation, 5 µg of proteins was applied to each lane of the SDS-PAGE gel under non-reducing conditions and Western blot analysis using TES101 mAb was carried out as described in Materials and Methods.

Results

Electrophoretic pattern of TEX101 after deglycosylation

N-Glycanase treatment

For enzymatic digestion of TEX101, Triton X-100 soluble testicular extracts were used for the experiments described below and probed with an anti-TEX101 mAb, TES101 (Kurita *et al.*, 2001). The Western blot pattern of the TEX101 showed two distinct bands at an apparent molecular mass of 38 kDa and faintly 34 kDa, respectively (Fig. 1, control lanes). These two bands were presumably due to the difference in glycosylation on TEX101, because a major size reduction in these bands occurred and they were observed as a distinct single band with a molecular mass of 20 kDa after a 48 h treatment with *N*-glycanase (Tarentino *et al.*, 1985), an enzyme known to cleave all types (high-mannose/hybrid, bi-, tri-, and tetra-antennary complex-type) of *N*-linked oligosaccharide (OS) chains (Fig. 1).

Endo-F2 treatment

When TEX101 was treated with endo-F2 (an endoglycosidase known to cleave high-mannose, certain types of hybrid OS and biantennary complex-type OS chains) (Plummer & Tarentino, 1991; Trimble & Tarentino, 1991), four distinct bands at molecular masses of approximately 37, 33.5, 29 and 24 kDa were observed (Fig. 1). It should be noted that intensity of these bands gradually increased in inverse proportion to their molecular mass. These results suggest that all four potential *N*-glycosylation sites found in TEX101 (Kurita *et al.*, 2001) are glycosylated, and most of these sites are occupied by endo-F2 sensitive OS chains.

Endo-H treatment

Subsequently, we treated TEX101 (testicular extract) with endo-H, which cleaves *N*-linked high-mannose/hybrid OS chains (Tarentino & Maley, 1974). Data from these experiments, presented in Fig. 1, showed that this treatment caused no major size reduction in TEX101 after a 48 h incubation period. However, a faint but distinct single band with a molecular mass of 20 kDa appeared after a long exposure to the film used to detect the ECL substrate (Fig. 2). The intensity of the bands was almost identical after 24/48 h of treatment with endo-H, suggesting that quite a low percentage of TEX101 molecules possess endo-H sensitive OS chains.

Effect of PIPLC on TEX101 expression in the testis

In adult mice, TEX101 is located mainly on the plasma membrane of spermatocytes, spermatids and the spermatozoa (especially in the tail portion) within the testis (Kurita *et al.*, 2001; Takayama *et al.*, 2005*a*). In addition, a previous study from our laboratories demonstrated two putative signal sequences at both

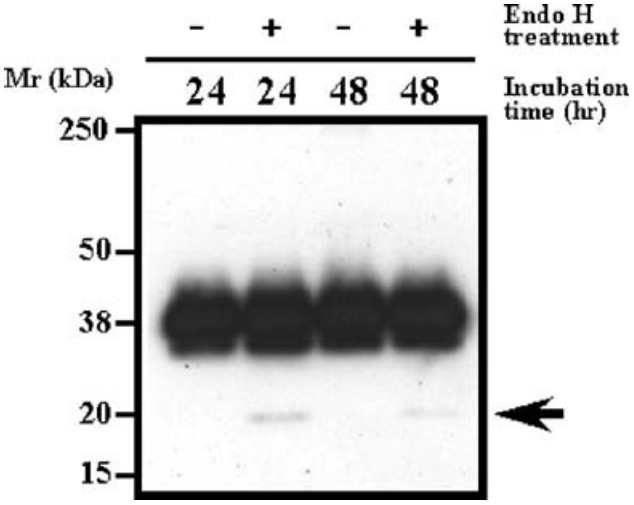

Figure 2 Western blot analysis of the testicular Triton X-100 fraction after endo-H digestion with TES101 mAb. A long time-exposure of the film was used to detect ECL substrate. Testicular Triton X-100 extract was digested with endo-H under identical conditions as described in the legend of Fig. 1. After Western blot analysis with TES101 mAb, ECL substrate was visualized by exposure of the film for 45 min. Arrow indicates a faint band observed at the position of approximately 20 kDa.

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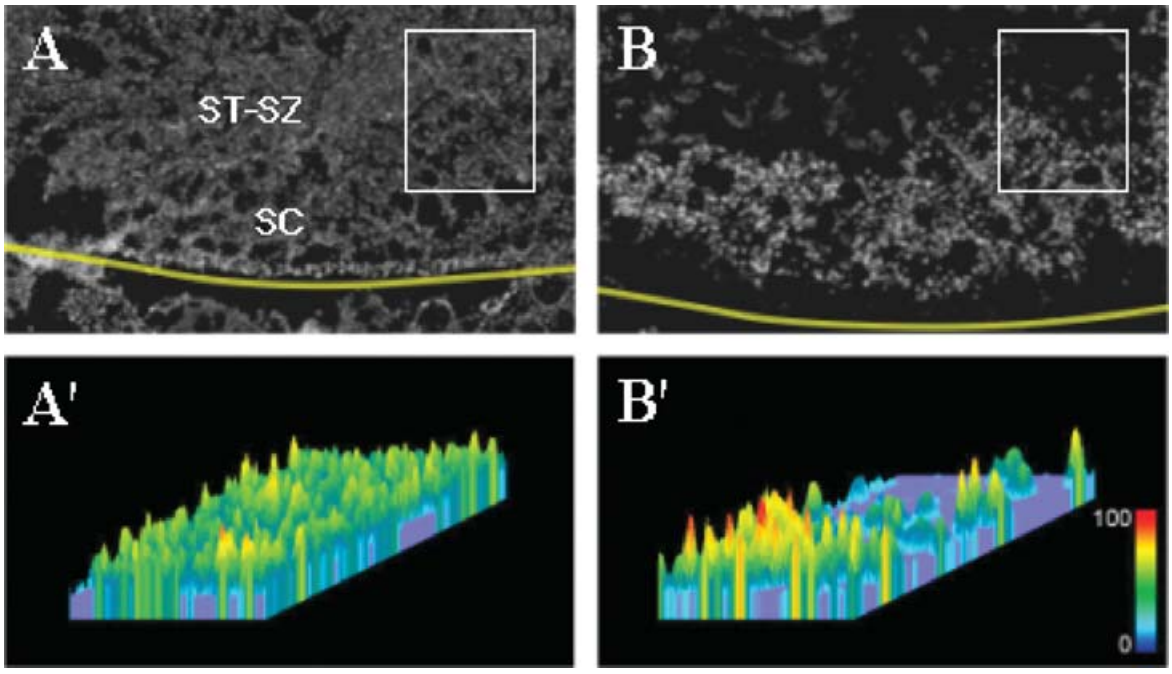

Figure 3 Immunofluorescence microscopic observation within mouse seminiferous tubule with the TES101 mAb after *in situ* PIPLC treatment. PIPLC treatment was carried out on the testicular tissue specimen, then TEX101 was visualized by an indirect immunostaining method as described in Materials and Methods. TEX101 immunofluorescence pattern of seminiferous tubule: (*A*) without PIPLC (control) and (*B*) with PIPLC treatment. Lower panels (*A′*, *B′*) indicate the relative fluorescence intensity images of the insets in (*A*) and (*B*), respectively. Yellow lines denote the boundaries of the testicular cords. SC, spermatocytes; ST, spermatids; SZ, spermatozoa.

the N- and C-terminals, which are typically found in GPI-anchored proteins (Yeh *et al.*, 1994; Udenfriend & Kodukula, 1995; Kinoshita *et al.*, 1997), within the deduced amino acid sequence of TEX101 by hydropathy plot analysis (Kurita *et al.*, 2001). These facts strongly suggest that TEX101 is a GPI-anchored protein (Yeh *et al.*, 1994; Udenfriend & Kodukula, 1995; Kinoshita *et al.*, 1997). To clarify whether the molecule is a GPI-anchored protein, we examined TEX101 immunoreactivity by treating it with PIPLC, an enzyme known to remove surface GPI-anchored proteins. First, testicular sections were treated with PIPLC, and then the expression of TEX101 was tested with a TES101 mAb probe. Within seminiferous tubules, TEX101 expression was abrogated, especially in the spermatids/spermatozoa-rich area (Fig. 3), although the reactivity to TES101 mAb appeared to be slightly enhanced in the area with spermatocytes containing TEX101 (Fig. 3).

Effect of PIPLC on TEX101 expression using transfectants

Transient expression of TEX101 in HEK293 cells and Western blot analysis

As a next step, we tried to establish a TEX101-transfectant, and examined the PIPLC sensitivity of TEX101 expressed on HEK293 cells. The cells were transfected with the pCD-TEX101 vector by a lipofectamine reagent. To verify the molecular mass of TEX101 expressed in the HEK293–TEX101 transfectants, cell extracts were analysed by Western blotting. As control experiments, parental HEK293 cells and mock transfectants were used that did not express TEX101 (data not shown). A major band with a molecular mass of 50 kDa that reacted with the TES101 mAb was detected in the HEK293–TEX101 transfectants (Fig. 4). The bands that were reactive with the mAb showed a higher molecular mass than native TEX101 (38 kDa) from the testicular extract (Fig. 4). Since HEK293 cells were established from human embryonic kidney (Graham *et al.*, 1977), the difference in molecular mass between native TEX101 and the molecule on the transfectants may have been due to the degree of TEX101 glycosylation. Indeed, treatment with *N*-glycanase against both TEX101 on the testis and HEK293 transfectants caused a size reduction to create nearly identical size bands (20 kDa; Fig. 4). In HEK293, although some OS chains on TEX101 showed resistance to *N*-glycanase treatment under our experimental conditions (Fig. 4), data suggest that TEX101 was at least partially expressed appropriately in HEK293–TEX101 transfectants at the protein level.

Treatment of PIPLC for HEK293–TEX101 transfectants

Attempts were then made to verify the molecular nature of TEX101 expressed on the transfectants by

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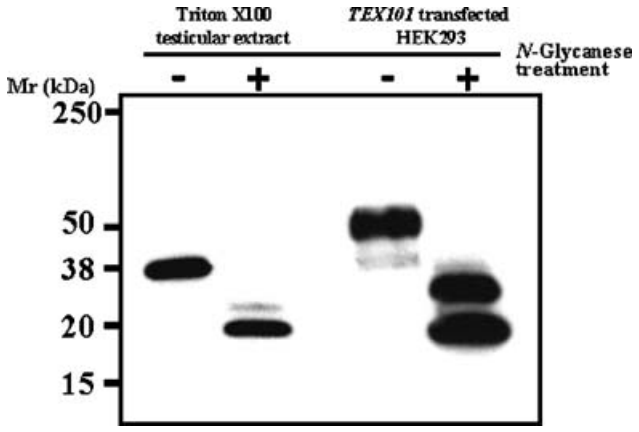

Figure 4 Verification of the TEX101 molecular mass expressed on TEX101/HEK293 cells. Solubilized cell extracts using *n*-octyl-β-D-glucoside from pCD-TEX101/HEK293 or testicular Triton X-100 extracts incubated with or without *N*-glycanase for 48 h at 37 °C. Samples containing 5 μg of proteins were applied to each lane of the SDS-PAGE gel under non-reducing conditions and Western blot analysis with TES101 mAb was carried out.

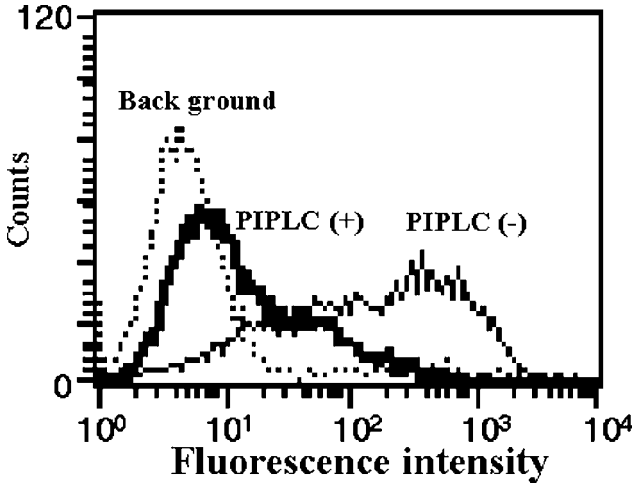

Figure 5 Analysis of TEX101 expression on TEX101 cDNA transfectant after treatment with PIPLC. The cells were incubated with (bold line) or without (thin line) PIPLC (1 U/ml) in PBS (pH 7.4) for 45 min at 37 °C, and then TEX101 expression on the cell surfaces measured by flow cytometry. For negative staining, the cells were reacted with a control mAb, 3H9 (dotted line).

flow cytometry. After 36 h transfection with pCD-TEX101, TEX101 expression was clearly observed on the cell surface of the transfectants (Fig. 5). The immunoreactivity decreased dramatically when the cells were treated with PIPLC, suggesting that TEX101 on the transfectant formed as GPI-anchored proteins. TEX101 expression on the mock transfectant or parental HEK293 cells was not detected by FACSCalibur (data not shown).

Discussion

In this study, we report further biochemical characterization of TEX101, a germ-cell-specific marker molecule that presumably plays an important role(s) in gametogenesis. The results of the TEX101 deglycosylation experiments using *N*-linked endoglycohydrolases suggest that: (1) TEX101 possesses *N*-linked OS units, and their average molecular mass is approximately 18 kDa; (2) all potential *N*-glycosylation sites (Asn-Xa.a.-Ser/Thr) found within TEX101 amino acid sequence (Kurita *et al.*, 2001) are glycosylated; (3) most of these four glycosylation site are occupied by endo-F2-sensitive OS units (high-mannose, certain types of hybrid OS, and biantennary complex-type OS chains); and (4) an extremely low population among TEX101 molecules possesses only the endo-H sensitive OS units (high-mannose/hybrid OS chain). Compared with the results obtained from endo-F2 and endo-H digestion of TEX101, the endo-F2 sensitive OS units on TEX101 do not contain high-mannose and hybrid OS units, since *N*-linked OS units on TEX101 almost lack sensitivity for endo-H (Fig. 1). Therefore, these results allow us to conclude that the majority of endo-F2 sensitive OS units on TEX101 are of the biantennary complex type, and very few of the OS units showing sensitivity for endo-H (Fig. 2) are of the hybrid type.

Although mature TEX101 was determined to be a protein with a molecular weight of 24 093 (Kurita *et al.*, 2001), Western blot analysis showed that TEX101 had a molecular mass of approximately 38 kDa under non-reducing conditions (Figs. 1, 2). Our preliminary Western blot analysis using anti-TEX101$_{39\text{-}53}$ peptide (DPGRTFNWTSKAEQC) detected a 38 kDa protein under both reducing and non-reducing conditions (Y. Araki, unpublished results). Therefore, we strongly speculated that this relatively large difference in the molecular mass was due to glycosylation of the peptide. Data presented here clearly demonstrate that TEX101 is highly glycosylated and that most OS units on TEX101 are *N*-linked carbohydrates, since de-*N*-glycosylation of TEX101 by endo-*N*-glycosidases produced a band of approximately 20 kDa (Figs. 1, 2), which is almost identical to (even lower than) the calculated molecular mass of the peptide bone of TEX101 molecule. However, we attempted but failed to qualitatively analyse *O*-linked OS units on TEX101 using *O*-glycanase or mild alkaline hydrolysis (β-elimination). We deglycosylated *N*-linked OS on testicular proteins extracted by Triton X-100 using *N*-glycanase. Then we treated the protein solution with *O*-glycanese in the presence of sialidase and α-L-fucosidase, or with 5 mM NaOH. However, the protein solutions in both cases were highly aggregated so that we were unable to separate them using SDS-PAGE. Presumably, this was due to the negative effects of large

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amounts of OS units being released from testicular proteins, since the viscosities of the sample solutions significantly increased after the *O*-deglycosylation treatments. Further studies using purified TEX101 will be necessary to analyse *O*-linked OS. Purification of TEX101 is presently under way in our laboratory.

The experiments using PIPLC suggested that TEX101 is expressed as a GPI-anchored protein, at least in part, because PIPLC treatments for testicular tissue sections or TEX101 transfectant caused a major reduction in TEX101 expression on the cell surface (Figs. 3, 5). Recently, the rat TEX101 homologue (TEC-21) was reported as a novel lipid raft-associated glycoprotein (Halova *et al.*, 2002). This molecule, originally identified on RBL-2H3, a rat basophilic leukaemia cell line (Barsumian *et al.*, 1981), was identified and characterized as a GPI-anchored protein on the surface of the cell line (Halova *et al.*, 2002). Although the expression pattern of rat TEC-21 is similar to that of mouse TEX101 (Halova *et al.*, 2002), no further information concerning the biochemical nature of TEC-21 on the testicular cells is available. The present study demonstrates, for the first time, evidence of the biochemical characteristics of TEX101 expressed as a GPI-anchored protein in the testicular germ cells.

Recently, the biophysical characterization of lipid raft domains on the sperm plasma membrane was reported: TEX101 was identified as a protein in the light buoyant-density, detergent-resistant fraction by proteomic analysis using tandem mass spectrometry (Sleight *et al.*, 2005). It should be noted, however, that TEX101 was detected mainly in the Triton X-100- and water-soluble fractions of the testicular extracts during spermatogenesis, as reported previously (Kurita *et al.*, 2001). This implies a possibility that TEX101 alters its biophysical forms during the process of spermatogenesis. Indeed, proteomic analysis reported by Sleight *et al.* used spermatozoa from cauda epididymis for their assay (Sleight *et al.*, 2005); we analysed TEX101 from testicular extracts for biochemical analysis (Kurita *et al.*, 2001). It has been reported that *TEX101* mRNA is expressed in spermatocytes as well as spermatids of the testis, but not in spermatogonia (Takayama *et al.*, 2005*a*, *b*). Although TEX101 proteins remained on the surface of testicular sperm, most were shed from the epididymal sperm in the caput epididymis (Takayama *et al.*, 2005*b*). At present, the physiological function of TEX101 in the process of gamete formation and maturation is still obscure; however, the biophysical character alteration of TEX101 during sperm formation may reflect its function(s) within gametogenesis.

In summary, we have further characterized the biochemical characteristics of TEX101 in mature mouse testis. TEX101 is highly *N*-glycosylated and forms, at least in part, as a GPI-anchored protein like TEC-21, the rat homologue of TEX101. The findings reported here provide a framework for further analysis and elucidation of the physiological importance of TEX101, a unique stage-specific marker molecule of germ cells.

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References

Anzai-Takeda, Y., Takeda, T., Sendo, F. & Araki, Y. (2005). Inhibition of cell spreading and stress fiber formation in CHO cells transfected with cDNA of a glycosylphosphatidylinositol-anchored glycoprotein, GPI-80. *Immunobiology* **210**, 1–10.

Araki, Y., Orgebin-Crist, M.-C. & Tulsiani, D.R.P. (1992*a*). Qualitative characterization of oligosaccharide chains present on the rat zona pellucida glycoconjugates. *Biol. Reprod.* **46**, 912–19.

Araki, Y., Vierula, M.E., Rankin, T.L., Tulsiani, D.R.P. & Orgebin-Crist, M.-C. (1992*b*). Isolation and characterization of a 25-kilodalton protein from mouse testis: sequence homology with a phospholipid-binding protein. *Biol. Reprod.* **47**, 832–43.

Barsumian, E.L., Isersky, C., Petrino, M.G. & Siraganian, R.P. (1981). IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* **11**, 317–23.

Graham, F.L., Smiley, J., Russell, W.C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59–74.

Halova, I., Draberova, L. & Draber, P. (2002). A novel lipid raft-associated glycoprotein, TEC-21, activates rat basophilic leukemia cells independently of the type 1 Fcε receptor. *Int. Immunol.* **14**, 213–23.

Kinoshita, T., Ohishi, K. & Takeda, J. (1997). GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. *J. Biochem.* **122**, 251–7.

Kurita, A., Takizawa, T., Takayama, T., Totsukawa, K., Matsubara, S., Shibahara, H., Orgebin-Crist, M.-C., Sendo, F., Shinkai, Y. & Araki, Y. (2001). Identification, cloning, and initial characterization of a novel mouse testicular germ cell-specific antigen. *Biol. Reprod.* **64**, 935–45.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.

Ohtake, K., Takei, H., Watanabe, T., Sato, Y., Yamashita, T., Sudo, K., Kuroki, M., Chihara, J. & Sendo, F. (1997). A monoclonal antibody modulates neutrophil adherence while enhancing cell motility. *Microbiol. Immunol.* **41**, 67–72.

Plummer Jr, T.H. & Tarentino, A.L. (1991). Purification of the oligosaccharide-cleaving enzymes of *Flavobacterium meningosepticum*. *Glycobiology* **1**, 257–63.

Sayama, K., Shiraishi, S., Shirakata, Y., Kobayashi, Y. & Miki, Y. (1991). Characterization of decay-accelerating factor (DAF). in human skin. *J. Invest. Dermatol.* **96**, 61–4.

Sleight, S.B., Miranda, P.V., Plaskett, N.W., Maier, B., Lysiak, J., Scrable, H., Herr, J.C. & Visconti, P.E. (2005). Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. *Biol. Reprod.* **73**, 721–9.

Suzuki, K., Watanabe, T., Sakurai, S., Ohtake, K., Kinoshita, T., Araki, A., Fujita, T., Takei, H., Takeda, Y., Sato, Y., Yamashita, T., Araki, Y. & Sendo, F. (1999). A novel glycosylphosphatidyl inositol-anchored protein on human leukocytes: a possible role for regulation of neutrophil adherence and migration. *J. Immunol.* **162**, 4277–84.

Takayama, T., Mishima, T., Mori, M., Jin, H., Tsukamoto, H., Takahashi, K., Kinoshita, K., Suzuki, M., Sato, I., Matsubara, S., Araki, Y. & Takizawa, T. (2005*a*). Sexually dimorphic expression of the novel germ cell antigen TEX101 during mouse gonad development. *Biol. Reprod*. **72**, 1315–23.

Takayama, T., Mishima, T., Mori, M., Ishikawa, T., Takizawa, T., Goto, T., Suzuki, M., Araki, Y., Matsubara, S. & Takizawa, T. (2005*b*). TEX101 is shed from the surface of sperm located in the caput epididymidis of the mouse. *Zygote* **13**, 325–333.

Tarentino, A.L. & Maley, F. (1974). Purification and properties of an end-β-*N*-acetylglucosaminidase from *Streptomyces griseus*. *J. Biol. Chem*. **249**, 811–17.

Tarentino, A.L., Gomez, C.M. & Plummer Jr, T.H. (1985). Deglycosylation of asparagine-linked glycans by peptide: *N*-glycosidase F. *Biochemistry* **24**, 4665–71.

Trimble, R.B. & Tarentino, A.L. (1991). Identification of distinct endoglycosidase (endo) activities in *Flavobacterium meningosepticum*: endo F1, endo F2, and endo F3. Endo F1 and endo H hydrolyze only high mannose and hybrid glycans. *J. Biol. Chem.* **266**, 1646–51.

Tsukamoto, H., Takizawa, T., Takamori, K., Ogawa, H. & Araki, Y. (2006). Genomic organization and structure of the 5′-flanking region of the TEX101 gene: alternative promoter usage and splicing generate transcript variants with distinct 5′-untranslated region. *Mol. Reprod. Dev.* (in press).

Udenfriend, S. & Kodukula, K. (1995). How glycosylphosphatidylinositol-anchored membrane proteins are made. *Annu. Rev. Biochem.* **64**, 563–91.

Yeh, E.T.H., Kamitani, T. & Chang, H.M. (1994). Biosynthesis and processing of the glycosylphosphatidylinositol anchor in mammalian cells. In *Seminar in Immunology*, vol. 6 (ed. J.D. Ashwell), pp. 73–80. London: Academic Press.

Yu, Y., Araki, Y. & Sendo, F. (2000). Tyrosine phosphorylation of a 34-kDa protein induced by crosslinking of a novel glycosylphosphatidylinositol-anchored glycoprotein (GPI-80). on human neutrophils that may regulate their adherence and migration. *IUBMB Life* **49**, 43–7.