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Molecular expression of Ly6k, a putative glycosylphosphatidyl-inositol-anchored membrane protein on the mouse testicular germ cells

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ABSTRACT

Ly6k, a putative mouse glycosylphosphatidyl-inositol (GPI)-anchored membrane protein is specifically associated with a unique germ-cell marker, TEX101. Although a human orthologue LY6K has been proposed as a novel cancer/testis antigen, its molecular nature is largely obscure, because its characteristics have been gleaned mainly from qualitative studies of gene structure. The aim of this study is to characterize molecular nature of Ly6k more precisely, especially, to focus on the molecular expression during testicular development. The present study have shown that: (1) *Ly6k* was strongly observed in testis, but faint expression was broadly noticed in other tissues; (2) *Ly6k* was weakly detected in testes from 18-day postcoitus to 1-day postpartum (dpp), with a plateau starting around 8-dpp; and (3) testicular *Ly6k* showed two-peak expression at around 14-dpp and 24-dpp, then exhibited stable expression from 6-week after birth onward. Western blot and immunohistochemical analyses revealed that Ly6k exists in at least two forms: a GPI-anchored form (17 kDa) and a water-soluble (non-membrane) form (12 kDa), and the 17-kDa mature form is expressed in the testicular germ cells beginning approximately 10 days after birth. This information is essential for the molecular classification of Ly6k, and may help uncover the detailed physiological role of Ly6k in gametogenesis, or cancer biology.

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1. Introduction

To analyze the molecular mechanism of spermatogenesis as well as the cellular function(s) of the spermatozoa, various marker molecules including germ cell surface proteins, have been identified and characterized [1,2]. We recently demonstrated that the mouse Ly6k was expressed in testes in association with TEX101, a unique germ-cell marker glycosylphosphatidyl-inositol (GPI)-anchored protein [3–7].

Ly6k is a relatively new member of the Ly-6-like protein superfamily, which is characterized by the presence of conserved cysteine residues and a putative GPI-anchoring site [8]. Elevated expression of LY6K, a human orthologue of mouse Ly6k, has been reported in testis and head/neck squamous cell carcinomas as well as in breast cancers [9,10]. In addition, there is growing evidence that LY6K is present in other cancers [11]. Therefore, this molecule is recently considered as a novel candidate cancer/testis antigen (CTA) [12,13]. Although LY6K is classified as a CTA mainly based on reverse transcription (RT)-polymerase chain reaction (PCR) analysis [11], little or no information concerning its precise tissue distribution especially within reproductive tissues has been published. Indeed, a recent study using a differential screening followed by signal sequence trapping demonstrated that Ly6k is a novel marker of mouse plasma cells [14].

To characterize Ly6k in detail, we developed a rabbit anti-mouse Ly6k polyclonal antibody (pAb) as a specific molecular probe [7]. Here, we further report the molecular characterization of Ly6k, with an emphasis on its expression profile in the testis during ontogeny, using the anti-Ly6k pAb [7] and a newly developed rat monoclonal antibody (mAb), mk34.

2. Materials and methods

2.1. Animals

Sexually mature ICR mice were purchased from Sankyo Labo Service (Tokyo, Japan). They were maintained and bred at our Animal facilities under 12L:12D conditions. Observation of the vaginal plug in the female mice after mating was considered as 0-day-postcoitus (dpc). For neonatal mice, the day when they were born was regarded as 1-day-postpartum (dpp). All animal experiments were conducted according to the guide for care and use of laboratory animals, Juntendo University.

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2.2. Preparation of antibodies

2.2.1. Production of anti-Ly6k rat mAb

2.2.1.1. Cells. HEK293 (CRL-1573) and SP2/0 cells (CRL-1581) were purchased from American Type Culture Collection (Rockville, MD). HEK293 cells and derived stable transfectants (see below) were maintained in DMEM supplemented with 10% fetal calf serum (FCS). SP2/0 cells and derived hybridoma clones were maintained in RPMI-1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol.

2.2.1.2. Construction of vectors. Full coding sequence of Ly6k was amplified from cDNA derived from splenic B cells by PCR with a sense primer, 5′-agactcgagGACCCGTGGACACGCTG-3′ (containing *Xho*I digestive sequence at the 5′-end), and an anti-sense primer, 5′-cctgcggccgcTTACAGCAGGCAGAGG-3′ (containing *Not*I digestive sequence at the 5′-end). The amplified cDNA fragment was subcloned into a pCAGGS1 vector [15] at the *Xho*I and *Not*I sites. To attach FLAG tag at the N-terminus of mature Ly6k, preprotrypsin signal sequence and FLAG peptide were amplified from a pFLAG-CMV1 vector (Sigma, St. Louis, MO) with a sense primer, 5′-taactcg agATGTCTGCACTTCTGATCC-3′ (containing *Xho*I digestive sequence at the 5′-end), and an anti-sense primer, 5′-atcggatccggtaccGTTAAC atcgatCTTGTCGTCATCGTC-3′ (containing *Bam*HI digestive sequence at the 5′-end), then subcloned into a pEF-BOS vector [16] using *Xho*I and *Bam*HI sites. The sequence coding putative mature Ly6k was amplified with a sense primer, 5′-agtggatccCTCA CCTGCCATGTGTGTGAG-3′ (containing *Bam*HI digestive sequence at the 5′-end), and the same anti-sense primer as described above. After digestion with *Bam*HI and *Not*I, the fragment was subcloned into the pEF-BOS vector downstream of FLAG sequence. The resultant vector (pF-mly6kaa21/pEF-BOS) expresses the protein consisting of preprotrypsin signal sequence, FLAG peptide and mature Ly6k from the N-terminus.

2.2.1.3. Establishment of stable transfectant cells expressing Ly6k on the cell-surface. HEK293 cells carrying a NF-κB reporter plasmid (293κB cells) were established by the co-transfection of p55IgκBLuc [17] with pBK-CMV (Stratagene, La Jolla, CA) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and following cultivation with G418. Positive single clones were screened with luciferase activity measured with the Steady-Glo Luciferase Assay System (Promega, Madison, WI). To establish 293κB cells expressing N-terminally FLAG-tagged Ly6k (293pF-mly6kaa21κB cells) and non-tagged Ly6k (293mly6kκB cells), 293κB cells were transfected by pF-mly6kaa21/pEF-BOS or mly6k/pCAGGS1 vector with a pBabePuro vector [18], respectively. After puromycin selection, stable clones were screened by the immunofluorescent staining with FLAG M2 mAb (Sigma) for 293pF-mly6kaa21κB cells and the supernatant of each hybridoma (see below) for 293mly6kkB cells followed by flow cytometry.

2.2.1.4. Preparation of a rat anti-mouse Ly6k mAb, mk34. Two Wister rats (Charles River, Yokohama, Japan) were immunized in their footpads with testicular water-insoluble fraction [3] as an emulsion with complete Freund's adjuvant at a total volume of 1.5 ml. One week later, cells were harvested from the draining lymph nodes of immunized rats. The harvested cells were fused with SP2/0 myeloma cells with PEG 1500 using a standard fusion protocol. Following HAT selection, culture supernatants were assayed by immunofluorescent test against 293pF-mly6kaa21κB and 293κB cells (negative control), and positive clones were isolated by the dilution planting technique. A mAb, named mk34 (IgG2a) reacted with 293mly6kκB cells, which excluded a possibility of the recognition to the artificially N-terminally tagged sequences. The mAb was purified from ascitic fluids by caprylic acid precipitation followed by DEAE ion exchange chromatography.

2.2.2. Other antibodies

Rabbit anti-mouse Ly6k pAb and an anti-TEX101 mAb (TES101) were produced and purified as previously described [3,7]. Other antibodies in this study was purchased from the following companies: Alexa Fluor 488- or 594-conjugated goat anti-rabbit IgG pAb and Alexa Fluor 488-conjugated anti-rat IgG pAb (Molecular Proves, Eugene, OR); horseradish peroxidase-conjugated goat anti-rabbit or rat IgG pAb, preimmune rabbit Ig and normal goat serum (DAKO, Glostrup, Denmark); and rat IgG2a (eBioscience, San Diego, CA).

2.3. RT-PCR analysis

RT-PCR analysis procedure was followed as reported previously [4,19]. PCR analysis for Ly6k transcript was performed in a 20 μl of 1 × Ex Taq™ buffer containing primers (0.5 μM each) specific for Ly6k transcripts (forward: 5′-CTCACCTGCCATGTGTGTGAGG-3′, reverse: 5′-TTACAGCAGGCAGAGGGCTGAGA-3′), dNTP mixture (0.2 mM each), and TaKaRa Ex Taq™ HS polymerase (0.05 U/ml)(TaKaRa). The sample was incubated at 94 °C for 1.5 min, and subjected to 30 cycles of PCR, denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extending at 72 °C for 30 s. After the reaction, the sample was continuously incubated at 72 °C for 7 min. As control studies, PCR analyses for *TEX101* and *β-actin* were performed according to the methods as reported previously [4,19]. The PCR products were separated by agarose gel electrophoresis, and then stained with ethidium bromide.

2.4. Quantitative PCR analysis

Total RNA from mouse testis was reverse transcribed by PrimeScript® RT reagent kit (TaKaRa). SYBR® Green method for quantitative PCR was carried out using SYBR® Premix Ex Taq™ (TaKaRa) with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. Following primer sets for Ly6k (forward: 5′-CAT GTGTGTGAGGCGCAGAAC-3′; reverse: 5′-ATTTTTGAACGTTTCTTC TAT-3′) or TEX101 (forward: 5′-TGGACTCTGGTCCAAAATACCT-3′; reverse: 5′-AATCCAGGAGAACTTTGCCAA-3′) were used to detect their molecular expression, respectively. Thermal cycling conditions comprised; an initial denature step at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s; dissociation stage at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

2.5. Immunohistochemistry

Testes and other organs from ICR mice were fixed in a PBS (pH 7.4) containing 4% PFA, and immunofluorescent localization of Ly6k was determined on cryostat sections as described previously [3–5,7]. The immunostained samples were examined with a BIOREVO BZ-9000 microscope system (KEYENCE, Osaka, Japan), and the images were analyzed with Adobe® Photoshop® elements 6 software (Adobe systems, San Jose, CA). Control sections received the same treatment, with the exception that the primary antibody was replaced with either preimmune rabbit Ig or rat IgG2a.

2.6. Western blot analysis

Organs from ICR mice were homogenized with a glass homogenizer in nine volumes of PBS (pH 7.2) containing EDTA-free Complete inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany). After sonication, the protein extracts were mixed with

an equal volume of 2 × SDS sample buffer [20] and boiled for 1 min to denature. After centrifugation, the supernatant was separated by the SDS–PAGE system [20] under non-reducing conditions. In some cases, tissue-extracts of Triton-X100®-soluble (TS) fraction and water-soluble (WS) were prepared according to the method as described previously [3,5]. The molecular mass of the antigen reactive with the primary antibodies was determined by enzyme immunostaining of the protein after blotting to a PVDF membrane from the SDS–PAGE gel according to the standard method [3,5].

3. Results and discussion

We primarily used RT-PCR analysis to examine the precise localization of Ly6k within several organs. Although TEX101 (a Ly6k-associated molecule)[5,7] is expressed on only germ cells in both male and female gonads during gametogenesis [3,4], *Ly6k* was broadly detected in the organs examined (strongly in the testis; weakly in the lung, spleen, and stomach; faintly in the kidney, brain, and heart), whereas no obvious positive signal was observed in the liver or intestine (Fig. 1A). In reproduction-related organs, *Ly6k* mRNA was strongly observed in testes (positive control), whereas relatively weak signals were observed in other reproductive organs, including the epididymis, ovary, and uterus (Fig. 1B).

At the protein level, our previous study showed that a strong staining was observed in testicular cryosections fixed with 4% PFA using the anti-Ly6k pAb [7]. In other organ, clear, but partial immunoreactivity was observed in the brain (cerebellum), stomach, lung, and spleen; the immunopositive signals were detected in the axonal regions of the cerebellum (Fig. 2A-a), the serosa of the stomach (Fig. 2A-d), and patches of cells in the lung and spleen (Fig. 2A-b and A-e). However, significant immunoreactivity was not observed in the kidney, liver (Fig. 2A-c and A-f), heart, or intestine (data not shown). These results indicated that immunohistochemical analyses using the anti-Ly6k pAb did not always parallel those of RT-PCR analyses for the tissues examined. In addition, no specific immunoreaction was observed at an appropriate molecular mass (17 kDa) for Ly6k by Western blot analysis of these mouse organs (except for the testis) (data not shown). Positive signals were also observed in the ovary, uterus, and epididymis (Fig. 2B). In the ovary, Ly6k was detected within secondary follicles (both granulosa cells and oocytes), but the signal was faint compared to that of Ly6k in the testicular germ cells (Fig. 2B-a and B-b). Faint and diffuse immunoreactivity was also detected in the myometrium of the uterus, but not in the endometrium (Fig. 2B-c). Spermatozoa in the caput epididymis were positively for Ly6k (Fig. 2B-d). Further investigation showed that Ly6k expression was reduced on the spermatozoa in the duct of the corpus epididymis (Fig. 2B-e), and most of the spermatozoa in the cauda epididymis lacked Ly6k immunostaining (Fig. 2B-f), similar to our results for TEX101 [21]. However, specific Ly6k immunoreactivity at 17 kDa was not confirmed in the ovary, uterus, or epididymis by Western blot analysis (data not shown).

To examine the expression of Ly6k on the testis during ontogeny, we performed RT-PCR analysis of *Ly6k* in the testis from 18-dpc to 8-week after birth. Similar to *TEX101* [4], faint *Ly6k* expression was observed in testis isolated from 18-dpc and 1-dpp, and this appeared to plateau beginning at 8-dpp (Fig. 1C). To examine the *Ly6k* expression more precisely, we performed quantitative analyses by real time RT-PCR of the expression of *Ly6k* and *TEX101* from 1-dpp to 8-week after birth. The expression level was normalized to that of *β-actin* in each sample of testis; the normalized level of *Ly6k* expression in 8-week-old mouse testis was used as a standard. There were two peaks of *Ly6k* expression

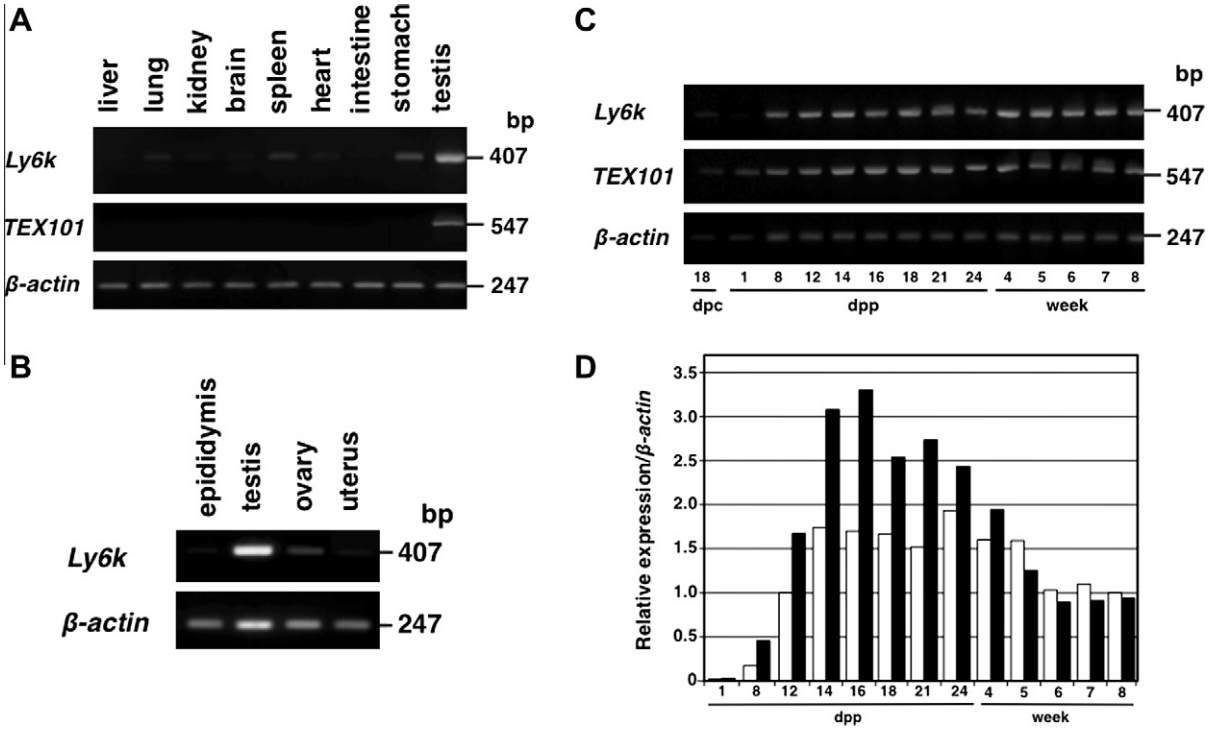

Fig. 1. Analysis of *Ly6k* mRNA expression by RT-PCR in mice. Expression profiles in the major organs (A), reproductive organs (B), and during testicular development (C). Total RNA was extracted from adult mice and subjected to RT-PCR analysis. RT-PCR products were separated on 2.0% agarose gels and visualized by ethidium bromide staining. Expressed positions in aliquots are at 407 bp (*Ly6k*), 547 bp (*TEX101* (a negative/positive control)) and 247 bp (*β-actin* (a quantitative internal control)). Quantitative RT-PCR analysis with SYBR Green method (D). Showing figure is one of typical experimental results of the real time PCR analysis. Relative expression value was normalized with *β-actin* expression and the normalized value of *Ly6k* expression at 8-week mouse testis is expressed as "1.0". Open and closed columns indicate the relative expression of *Ly6k* and *TEX101* at the each point of ontogeny, respectively.

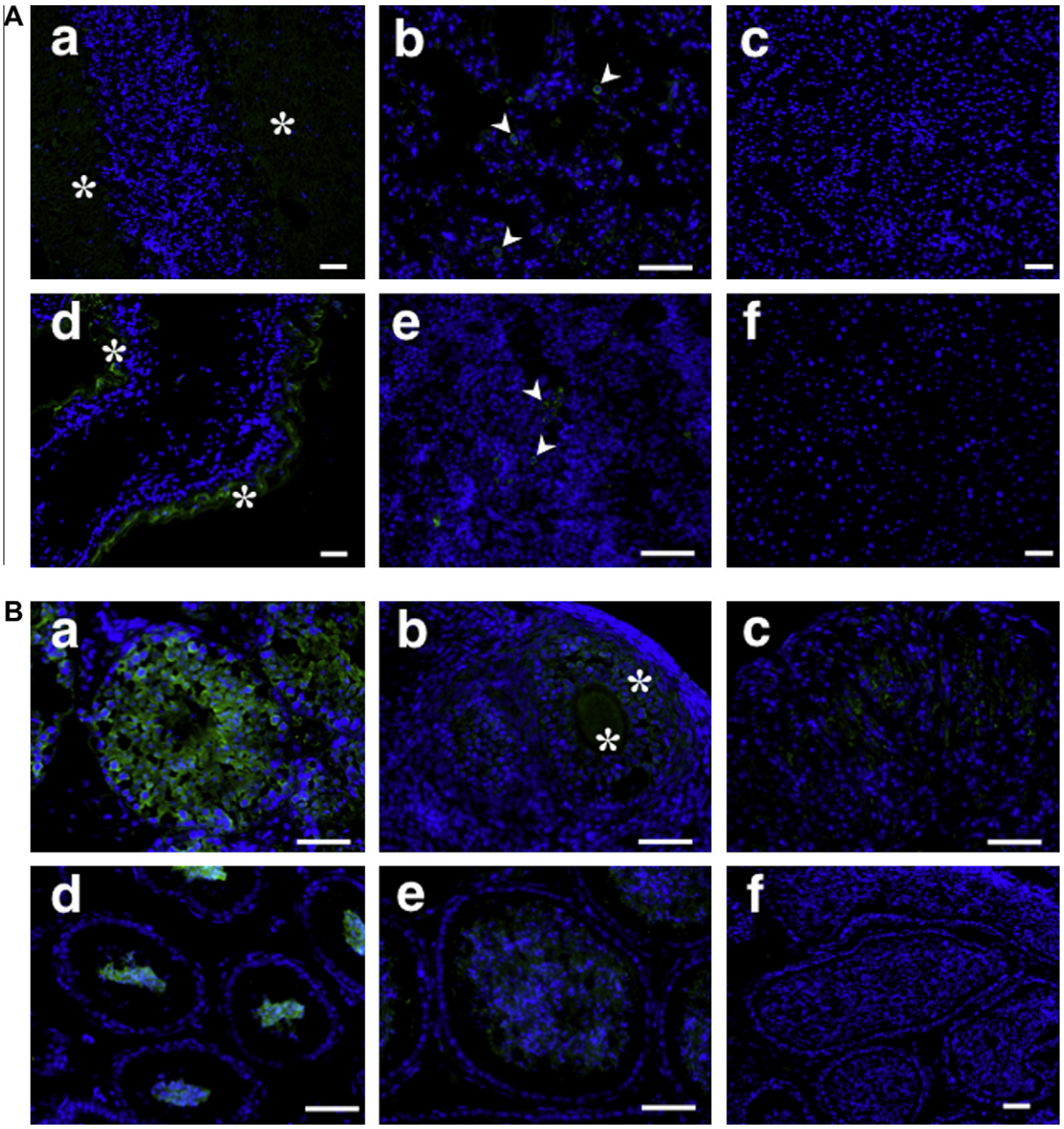

Fig. 2. Immunofluorescent analysis of Ly6k in sexually mature mouse organs fixed with PFA. Major organs (A): a, cerebellum; b, lung; c, kidney; d, stomach; e, spleen; f, liver; and reproductive organs from sexually mature mouse (B): a, testis; b, ovary; c, uterus; d, caput epididymis; e, corpus epididymis; f, cauda epididymis. Frozen sections were incubated with anti-Ly6k pAb, then visualized with Alexa Fluor 488-conjugated secondary antibodies (green), following nuclear counterstaining with DAPI (blue). Immunopositive regions are denoted by asterisks in A-a (the axon of cerebellum), A-d (serosa of stomach) and B-b (ovarian follicle; oocyte and granulosa cells). Scattered immunopositive cells in the tissues are highlighted by arrowheads (A-b and A-e). As expected, no immunopositive cell is observed in the liver (RT-PCR negative for *Ly6k*) (A-f), whereas the immunoreactivity is not found in the kidney (RT-PCR positive for *Ly6k*) (A-c). Bars: 50 μm.

at around 14- and 24-dpp, with stable expression beginning 6 weeks after birth (Fig. 1D). The two-peak expression pattern of *Ly6k* almost paralleled that of *TEX101* during testicular development after birth (Fig. 1D).

We next performed immunofluorescence analyses of testes from 18-dpc to maturity using the anti-Ly6k pAb. Prior to this study, we reported the TEX101 expression during testicular development [4] as follows: (1) by 8-dpp, prospermatogonia including neonatal-type undifferentiated spermatogonia [22,23] constitutively expressed TEX101; (2) from 10- to 12-dpp, the male germ cells had differentiated into adult-type spermatogonia (TEX101 was no longer detectable in these types of spermatogonia after this stage) rather than prospermatogonia, and TEX101-positive cells were observed occasionally in the testes (eventually these cells transformed into spermatogonia that lacked TEX101 expression); (3) from 12- to 14-dpp (after the start of spermatogenesis), TEX101 was detected on the surface of spermatocytes in the tubules; and (4) from 12-dpp through 28-dpp (i.e., the spermatogonia had differentiated into spermatozoa; cells at all stages of spermatogenesis, including spermatozoa may be observed at 28-dpp), TEX101 expression was essentially the same as that shown previously by immunostaining in the mature testis [3]. Unlike Tex101, the anti-Ly6k pAb showed weak immunoreactivity against the testicular cryosections from 18-dpc embryos (Fig. 3A).

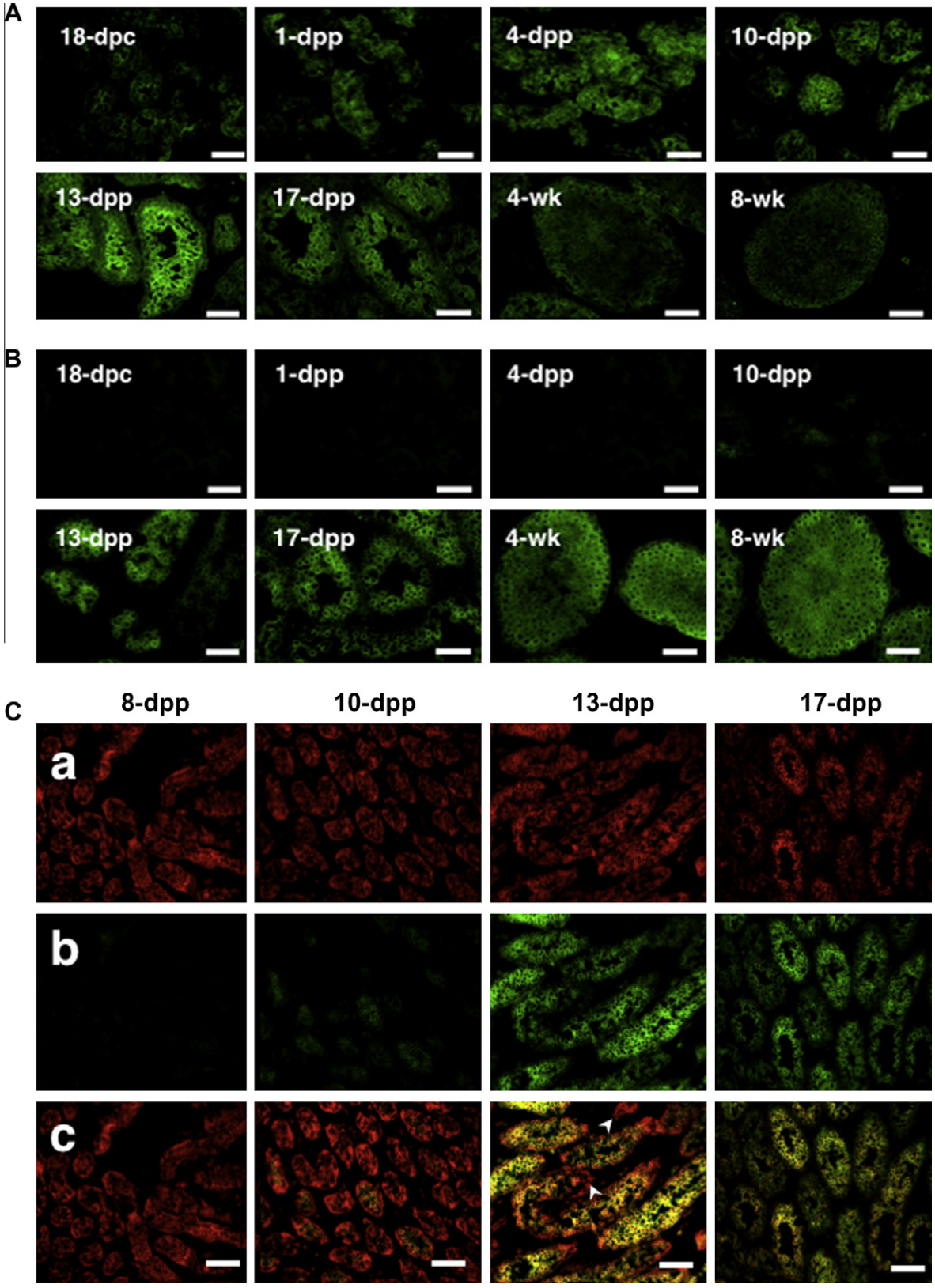

Fig. 3. Ly6k immunohistochemistry of the developing mouse testis. Stained with the anti-Ly6k pAb (A), and with a mAb for Ly6k (mk34) (B) of testes isolated from 18-dpc to 8-week old, and double immunostaining of the developing testis (C). The fluorescent staining patterns of the pAb (red) (a), mk34 (green) (b), their overlay (c). Note only the pAb reactive regions of the testicular seminiferous tubules from 13-dpp-old mouse (arrowheads). Bars: 40 μm.

From 18-dpc, the strength of the signal gradually increased by around 10- to 13-dpp, and the surface as well as cytoplasm of the germ cells were immunopositive at this stage (Fig. 3A). After 17-dpp, the distribution of Ly6k was mainly observed on the surface of germ cells (Fig. 3A), like in the seminiferous tubules of sexually mature mice [7].

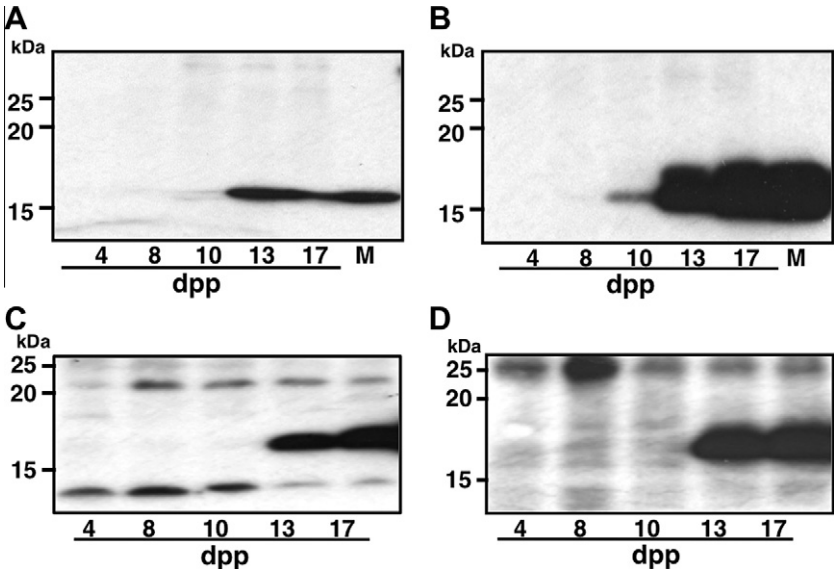

Fig. 4. Western blot analyses of testicular extracts during development. Immunoreactivity of testicular TS fraction with the pAb (A), or mk34 (B). No significant immunoreaction is observed in the TS testicular extracts before 8-dpp. Immunostaining pattern of testicular non-TS fraction with the pAb (C), or mk34 (D). An additional band at the apparent molecular mass of 12 kDa (indicated by an arrow) is noticed in the samples only when the pAb was used as a detective probe.

In this study, we further established the novel mAb against mouse Ly6k, mk34 by immunizing rats with a mouse testicular water insoluble fraction (containing Triton X-100-soluble and insoluble fractions) to investigate the molecular characteristics of Ly6k. During the course of our immunohistochemical experiments, we unexpectedly found that the immunostaining pattern of mk34 was different than that of the anti-Ly6k pAb. The pattern of immunoreactivity for mk34 in sexually mature mouse (8-week or older) testicular sections was similar to that for the anti-Ly6k pAb (Fig. 3AB). However, obvious positive staining for mk34 was not observed in the testicular specimens prepared from 18-dpc to ~10-dpp (Fig. 3B). From 10-dpp, the level of immunofluorescence gradually increased, and all seminiferous tubules became immunopositive by 17-dpp (Fig. 3B).

Double staining with the anti-Ly6k pAb and mk34 confirmed the difference in immunoreactivity against the frozen testicular sections. In similar to the results showing in Fig. 3A, the anti-Ly6k PAb showed immunoreactivity against the seminiferous tubules throughout the investigation period (Fig. 3C-a). By 8-dpp, the prospermatogonia exist in the seminiferous tubules. Before this stage, immunoreactivity of mk34 was not observed in the testis (Fig. 3C-b). From 10- to 12-dpp, the male germ cells were readily identified by nuclear staining using Hoechst 33342 or DAPI as adult-type spermatogonia (i.e., type A, intermediate, and type B spermatogonia) rather than prospermatogonia. Around this stage, mk34 showed immunoreactivity against a part of seminiferous tubules (Fig. 3C-b). It should be noted that the cytoplasm of the germ cells that were positive for the anti-Ly6k pAb but negative for mk34, were still observed in the seminiferous tubules at 13-dpp (Fig. 3C-c). After 17-dpp, the immunopositive regions for the both pAb and mAb were almost entirely overlapped in the germ cells (Fig. 3C-c).

Because a different immunoreactivity between the pAb and mk34 was observed on the seminiferous tubules during sexual maturation (Fig. 3), we further investigated concerning immunoreactivity of both antibodies causing this discrepancy. During a course of study, we noticed that the immunoreactive pattern of the anti-Ly6k pAb in the seminiferous tubules was different between before 13-dpp and after 17-dpp. From 18-dpc to 13-dpp, the anti-Ly6k pAb showed immunoreactivity against both the surface and cytoplasm of the germ cells, whereas the immunopositive staining of the pAb was mainly observed on the cell surfaces (Fig. 3A). These results suggested that Ly6k might exist as a non-membrane form as well as a membrane form in the male germ cells before 13-dpp. In general, non-membrane and membrane types of proteins were separated into WS and detergent-soluble fractions, respectively [3,7]. We examined the reactivity of detergent-fractionated testicular extracts from sexually immature mice to pAb and mk34 by Western blot analysis. We first analyzed testicular TS membrane fractions, because Ly6k has been predicted to be a GPI-anchored membrane based on its primary structure [8]. In addition, we previously demonstrated that Ly6k was predominantly detected in a TS fraction isolated from sexually mature mice [7]. Although the sensitivity (affinity) of the antibodies for the TS fraction was somewhat different, both antibodies detected a band at an apparent molecular mass of 17 kDa equally from 10-dpp onward (Fig. 4AB).

Attempts were then made to examine non-TS testicular fractions (WS extracts) by Western blot analysis using the pAb or mk34. Although their staining patterns against the 17-kDa band were not significantly different, an additional band at the 12 kDa was detected in the non-TS samples from 4- to 17-dpp mice for the pAb (Fig. 4C), but not mk34 (Fig. 4D). Interestingly, the 17-kDa band from the non-TS fraction was clearly detected beginning 13-dpp, whereas expression of the 12-kDa band was in inverse proportion to that of the 17-kDa band (Fig. 4C). The anti-Ly6k pAb was immunoreactive against the proteins corresponding to both 17-kDa and 12-kDa bands after affinity purification with recombinant full-length Ly6k (data not shown). Thus, the 12-kDa form may lack the binding region for mk34, making it a non-membrane-binding form of Ly6k. At present, however, there is no experimental evidence to confirm a splice variant, which would produce a truncated form of Ly6k by the capsite hunting method [24] from mouse testis capsite cDNA dT [19]. In addition, RT-PCR analysis clearly showed a transcript of 407 bp (coding region of full-length Ly6k = 462 bp) in the testis before 8-dpp (a specific period when only the 12-kDa band was detected). Therefore, the 12-kDa protein is likely produced from full-length Ly6k by posttranslational modification. Additional studies using mass spectrometry after immunoprecipitation with pAb will be necessary to clarify the issue, although difficulty is expected in collecting enough protein from neonatal mice testes.

4. Conclusions

The present study involved the further molecular characterization of Ly6k, a putative GPI-anchored protein. There are at least two forms of the molecule, a detergent-soluble membrane-bound form and water-soluble form, each of which is expressed in a characteristic way during testicular development after birth. Our data will help lay the groundwork for the molecular classification of the Ly6k, and should facilitate an analysis of the detailed physiological role of Ly6k in gametogenesis or cancer.

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