Journal of Reproduction and Development, Vol. 56, No. 6, 2010

—Original Article—

Analysis of Prolactin Gene Expression and Cleaved Prolactin Variants in the Mouse Testis and Spermatozoa

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Abstract. Prolactin (PRL) has long been known to be a hormone responsible for mammary gland development and lactation in females, whereas its role in males is still unclear. Thus, we investigated male mouse (m) PRL protein and mRNA expression in spermatozoa at various differentiation stages in the testes. Quantitative RT-PCR and *in situ* hybridization detected the expression of PRL not only in Leydig cells but also in germ cells, in particular in spermatogonia. The nucleotide sequence of testis PRL mRNA was the same as that in the pituitary. The mPRL was detected in Leydig cells and in round and elongated spermatids of the testes by immunohistochemistry. Immunoblotting detected 2 forms of mPRL in the testes, one form was 23-kDa PRL, and the other form was smaller than full-length PRL. Based on these results, we focused on N-terminal cleaved PRL to determine its involvement in spermatogenesis. Immunohistochemistry using two sets of antibodies, one that recognized full-length PRL and N-terminal cleaved PRL and another that recognized full-length PRL and C-terminal cleaved PRL, suggested that intact PRL was localized in the nucleus of round spermatids, while N-terminal cleaved PRL variants were localized in the Golgi apparatus of the sperrmatid nuclei of round spermatids, cytoplasms of elongated spermatids and in the spermatozoa tails. These findings suggest that PRL is ectopically expressed in the spermiogenesis and spermatogenesis and that cleaved PRL variants were localized in the Golgi apparatus of spermatids and in spermatozoa tails.
Key words: Ectopic expression, Mouse, Prolactin, Testis

(J. Reprod. Dev. 56: 567–574, 2010)

The various functions of prolactin (PRL) in vertebrates include the growth and differentiation of the mammary epithelium, lactation in mammals, osmoregulation and parental behavior in teleosts, amphibian development, broodiness in hens, crop sac production in pigeons and immunoregulation [1].

Moreover, many studies generally suggest that PRL positively modulates testicular functions in several ways, as follows: PRL is involved in the upregulation of LH receptor on Leydig cells [2, 3], in the increase in FSH receptors in Sertoli cells [4], and in the meiosis of germ cells [5]. In contrast, none of the parameters or functions of the male reproductive organs are affected in PRL knockout (KO) or PRL receptor (PRLR) KO mice [6, 7]. Thus, the exact role of PRL in the regulation of testicular function is still unclear. It is unknown why the lack of PRL signaling in PRLR KO and PRL KO mice affects the reproductive function of the male mouse in such a way that the expected results are marginal compared with those obtained in hypophysectomized or PRL deficient hereditary dwarf mice or even absent. Perhaps normal or near-normal reproductive function in PRLR KO and PRL KO animals is due to compensatory mechanisms and/or alternate signaling pathways (e.g., growth hormone) that are absent in both hypophysectomized animals and hereditary dwarf mice [8].

Additively, these apparent discrepancies may be due to the existence of structural variants of PRL in the testes. Recent studies have shown that PRL exists in several molecular forms, some arising from genetically determined factors and others from posttranslational modifications. The genetic variants include variant genes and splice variants of PRL [9]. On the other hand, the posttranslationally modified variants include cleaved, glycosylated and phosphorylated PRL [9]. Targeted disruption of either PRL or PRLR may be ineffective for these PRL variants. For instance, mutation of PRL by a targeted insertion that truncated the protein at residue 117 resulted in an intact N-terminal PRL fragment of 11 kDa [10]. In addition, these PRL fragments do not appear to signal through PRLR but rather through a distinct, endothelial cell surface receptor [11] whose activity would be unaltered by targeted deletion of the PRLR.

Furthermore, functional analysis of PRL is complicated by the existence of extrapituitary PRL. Extrapituitary PRL mRNA expression is not an uncommon phenomenon, and the various tissues that produce PRL include the placenta, nipple, spleen, lachrymal gland, skin and brain [12–15]. Roux M *et al.* [16] showed that PRL immunohistochemistry was detected in the interstitial tissue, Sertoli cells and germ cells in adult and prepubertal animals. That study, however, did not clarify whether the PRL was produced or only stored by the testis and nor did it clarify its function. The function of extrapituitary PRL was very interesting, but the difference of those functions was not clear in a PRL –/– mouse, which possessed neither pituitary gland nor extrapituitary PRL [10]. In general, it is thought that extrapituitary PRL compensates for deficiency in pituitary PRL and when a large quantity of PRL is

Received: September 16, 2009
Accepted: June 15, 2010
Published online in J-STAGE: July 20, 2010

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needed [17, 18]. However, the relative contribution of the pituitary and extrapituitary PRL is unknown.

Based on these data, the present study focuses on ectopic PRL expression and PRL variants in the mouse testis and spermatozoa.

Materials and Methods

Animals

The experimental design of this study complied with the guidelines for animal experiments of our institution. We housed ICR mice under controlled temperature conditions (22 ± 2 C) in an artificially illuminated room (12-h light/12-h dark cycle). Food and tap water were freely available. At 70–90 days of age, females were mated with males, and the day a vaginal plug was present was designated as day 0 of pregnancy. The day of birth was designated as infant day 0. Mice were sacrificed by ether anesthesia at 10, 20, 30, 40 and 120 days after birth. The pituitary glands and testes were removed and stored at –80 C until use.

Antibodies

We used three types of PRL antibody. One antibody was rabbit antiserum against recombinant mouse PRL (mPRL), which recognizes all types of PRL (PRL Ab). The second antibody was against oligopeptide corresponding to the 104–116 aa of mPRL (N-PRL Ab). The third antibody was against oligopeptide corresponding to the 174–186 aa of mPRL (C-PRL Ab). Figure 1A provides a schematic diagram of the antibodies. The crude peptide was purified by reverse-phase high-performance liquid chromatography with a 0–60% linear gradient of CH3CN in 0.1% trifluoroacetic acid. Purification of the peptide was confirmed by measuring its molecular mass by mass spectrometry. The antibodies were raised in a chicken immunized with the N-PRL and C-PRL peptide (Japan-lamb, Hiroshima, Japan). Immunoblot analysis using each antibody against the recombinant mPRL, which was truncated by Cathepsin D, revealed the specificity of each Ab to cleaved PRL as follows. PRL Ab recognized approximately 23-kDa, 16-kDa and 6-kDa bands of PRL. However, N-PRL Ab recognized approximately 23-kDa and 16-kDa bands but not 6-kDa PRL. On the other hand, C-PRL Ab recognized approximately 23-kDa and 6-kDa bands but not 16-kDa PRL. Therefore, comparison of each reaction among the three antibodies showed differences of PRL and PRL variants (Fig. 1B). In addition, the specificity of the immunoreactivity was checked using of an absorption test by preincubating each antibody with the recombinant mPRL (1 μg/ml).

The anti-Golgi 58K protein monoclonal antibody (Sigma-Aldrich Japan, Tokyo, Japan), which specifically recognizes the Golgi apparatus, was used for the Golgi marker. Secondary antibodies of biotin-conjugated goat anti-rabbit IgG and rabbit anti-chicken IgG (Vector Laboratories, Burlingame, CA, U.S.A) were used for immunohistochemistry and immunoblotting. Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-chicken IgG and Alexa Fluor 488 goat anti-mouse IgG (Life Technologies Japan, Tokyo, Japan) were used for indirect immunofluorescence histochemistry.

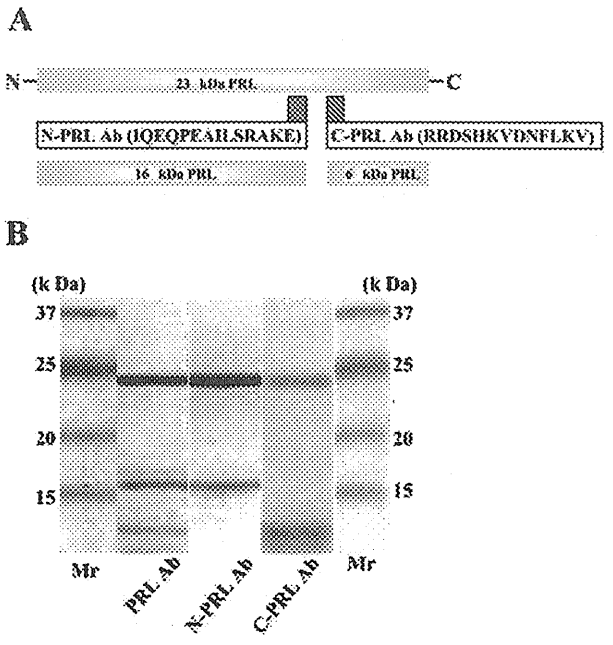

Fig. 1. A: Schematic diagram of the antibodies. N-PRL Ab recognizes $PRL_{103-116}$ peptide, which includes full-length PRL and N-terminal cleaved PRL. C-PRL Ab recognizes $PRL_{174-186}$ peptide, which includes full-length PRL and C-terminal cleaved PRL. B: Specificity of the antibodies. Immunoblotting against the recombinant mPRL, which was truncated by Cathepsin D. PRL Ab recognized approximately 23-kDa, 16-kDa and 6-kDa bands. The N-PRL Ab did not react to an approximately 6-kDa band, and the C-PRL Ab did not react to an approximately 16-kDa band.

Tissue preparation

For *in situ* hybridization and immunohistochemistry, mouse testes were fixed in Bouin's fluid for 6 h and embedded in paraffin. Sections 6 μm thick were cut on a microtome and mounted on 3-aminopropyltriethoxysilane-coated slides. Serial sections were prepared to compare the immunoreactions of different PRL antibodies.

Sperm collection

Spermatozoa were collected from the cauda epididymis of mature male mice. The distal portion of each epididymis was cut using a blade, and a dense sperm mass was squeezed out of the epididymis. Sperm were suspended in TYH medium under oil in a dish. These spermatozoa were incubated for 60 min at 37 C under 5% CO_2 in air for capacitation. The spermatozoa were then diluted and counted using a hemocytometer. The final concentration of spermatozoa was adjusted to 2×10^6 spermatozoa/ml in TYH medium.

Real-time RT-PCR

Real-time RT-PCR was performed using Taq Man Universal PCR Master Mix and an ABI Prism 7500 Sequence Detector (Life

Technologies Japan) according to the protocol of the manufacturer. The TaqMan Gene Expression Assay IDs were Mm00599949_m1 (PRL) and Mm99999915_g1 (GAPDH), respectively.

Nucleotide sequencing

We estimated the concentration of total RNA isolated from 100 mg of testis using an RNA extraction Kit (ISOGEN, Wako Pure Chemical Industries, Osaka, Japan). First strand cDNA synthesis from 1 μg of total RNA was performed using a QuantiTect Reverse Transcription Kit (QIAGEN Japan, Tokyo, Japan) according to the protocol of the manufacturer. The cDNAs were then subjected to PCR amplification by 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 74 C for 1 min using KOD DNA Polymerase (TOYOBO, Osaka, Japan) and a GeneAmp PCR system 9600 (Bio-Rad Laboratories, Tokyo, Japan). The PRL primers corresponded to the PRL cDNA (accession No. P06879). For PRL, the primers were as follows: sense, 5'-CTGCCAATCTGTTCCGCTG-3'; antisence, 5'-AGAGATGGACTGAAT GTGGGT-3'.

We purified a testis ectopic PRL product in Nucleo Spin Extract II (Macherey-Nagel, Duren, Germany), and cDNA was subcloned into the *BamH* I-*Sal* I site of Bluescript pSK (-) plasmid. The nucleotide sequence was reacted with a BigDye Terminator v3.1 Cycle Sequence kit (Life Technologies Japan), and the product was purified in BigDye XTerminator (Life Technologies Japan). An ABI PRISM 3100 Genetic Analyzer and the Sequence Scanner software ver1.0 (Life Technologies Japan) were used for analysis, and the results were compared with an already known pituitary PRL sequence.

In situ hybridization

In situ hybridization was performed as described previously with minor modifications [14]. In brief, the sections were pretreated with proteinase K (10 ng/ml) solution, 0.2 N-HCl and 0.1 M triethanolamine before hybridization. Hybridization proceeded with the biotin-labeled PRL cDNA probe (5'-CTTCCGGAGGGAC TTTCAGGGCTTG-3') for 16 h at 42 C. The specificity of this cDNA probe was demonstrated in our previous study [14]. Negative control sections were incubated with only hybridization buffer. After hybridization, sections were washed twice in 2× SSC and 0.2× SSC. Hybridized biotin-conjugated cDNA probes were detected with the horseradish peroxidase-conjugated anti-biotin antibody for 1 h and then incubated with an ABC Standard kit (Vector Laboratories) for 30 min. Immunohistochemical reactions were visualized using a Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific, Yokohama, Japan). Sections were counterstained with Mayer's hematoxylin.

Immunoblotting

Pituitaries and testes were disrupted ultrasonically in tissue protein extraction reagent and a Halt Protease Inhibitor Cocktail Kit (Thermo Fisher Scientific) and separated by centrifugation at 15,000 *g* for 10 min. Equivalent amounts of sample buffer (0.5 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol (2-ME) and 0.01% (w/v) bromophenol blue) were added to the supernatants, and the samples were then heated 95 C for 5 min. The samples were resolved by 15% SDS-PAGE. Separated proteins were electrophoretically transferred to Immobilon-P membranes (Nihon Millipore, Tokyo, Japan) and incubated with the primary antibodies PRL Ab, N-PRL Ab and C-PRL Ab (1:10000). After washing with TBS, membranes were incubated with secondary antibodies, biotin-conjugated goat anti-rabbit IgG, and visualization were performed as for *in situ* hybridization.

Immunohistochemistry

The sections were autoclaved in 10 mM sodium citrate (pH 6) at 115 C for 5 min to do retrieve the antigen. After treatment with 0.1% Triton X-100 for 10 min, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Furthermore, non-specific binding of antibody was blocked with Block Ace (Summit Pharmaceuticals, Tokyo, Japan) for 1 h at room temperature. The sections were then incubated with primary antibody at 4 C overnight. Control sections were incubated with Block Ace instead of each primary antibody (1:10000), respectively. After washing with PBS, sections were incubated with the secondary antibody, rabbit anti-chicken IgG (1:500, Vector Laboratories), for 1 h. Detection and visualization were performed as for *in situ* hybridization. Sections were counterstained with Mayer's hematoxylin or periodic acid Schiff (PAS).

Immunofluorescence histochemistry

Sections, or sperm on slides, were pretreated by autoclaving (sections only) and incubation in 0.1% Triton X-100 and Block Ace and then incubated with the primary antibody N-PRL Ab (1:10000) and the anti-Golgi 58k protein monoclonal antibody (Sigma-Aldrich Japan), which specifically recognizes the Golgi apparatus, in the same manner as for immunohistochemistry. After rinsing with PBS, the samples were incubated for 1 h with secondary antibodies, Alexa Fluor 568 goat anti-rabbit IgG, Alexa 568 goat anti-chicken IgG and Alexa Fluor 488 goat anti-mouse IgG (1:500, Life Technologies Japan).

The sections were mounted and counterstained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). Slides were examined under an LSM510 Confocal Laser Scan Microscope (Carl Zeiss Japan, Tokyo, Japan).

Results

Testis PRL expression and nucleotide sequence analysis

We performed real-time PCR for PRL from the testes of 7- to 120-day-old mice. PRL was ectopically expressed in testes days of age; at the day 7, the level of expression was especially high level.

The quantity of each testis PRL PCR product compared with a pituitary PRL PCR product was very small. The proportion of amplified RT-PCR PRL products in the male adult testis vs. those in the pituitary was 1:9.4 (data not shown).

Then, we determined the testis PRL nucleotide sequence and confirmed that the sequence was the same as that of a pituitary PRL (data not shown).

Localization of PRL in the mouse testis

In situ hybridization for PRL mRNA was performed using a biotin-labeled PRL cDNA probe to determine the site of PRL

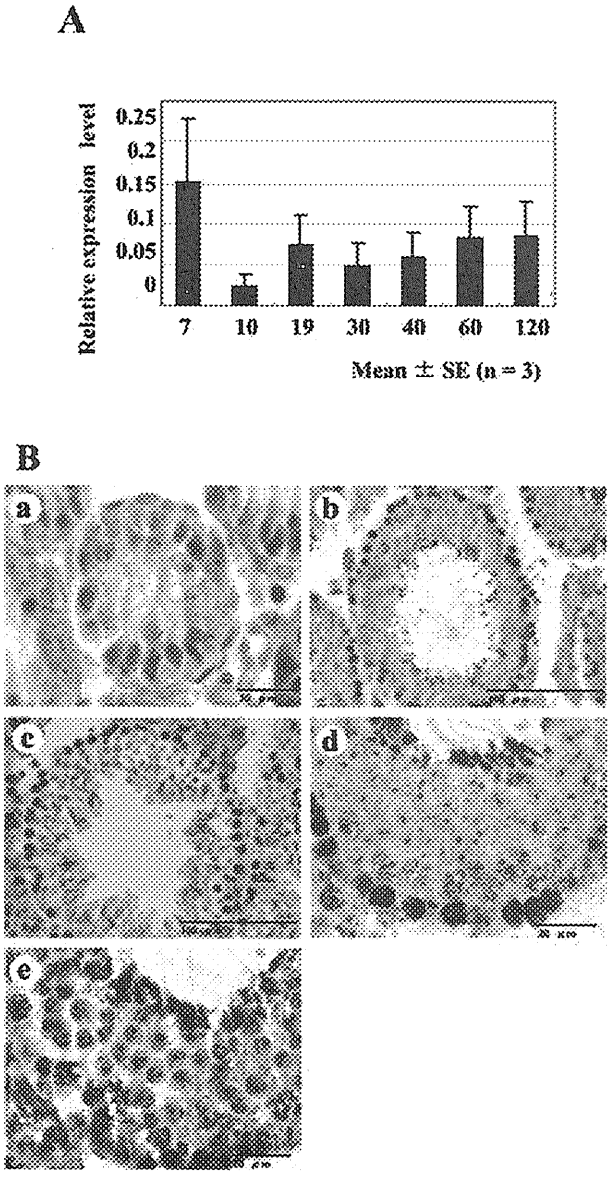

Fig. 2. A: PRL expression in the mouse testis. Real-time PCR analysis of PRL in the testis. The longitudinal axis shows the level of PRL expression relative to GAPDH expression. Data is presented as the mean ± SE (n=3) and expressed in terms of arbitrary units per ng total RNA in the reverse transcription reaction. The horizontal axis shows the ages of the testes in days, 7, 10, 20, 30, 40, 60 and 120. B: *In situ* hybridization for PRL mRNA in the testis. Panels a–e show the testis on days 10, 40 and 120, respectively. Panel d shows day 120 and higher magnification images. Intense immunoreactivity to PRL mRNAs was observed in the spermatogonia, pachytene stage spermatocytes and preleptotene stage spermatocytes. Weak signals from PRL mRNAs were observed in the spermatids from 40 (b)- and 120 (c, d)-day-old mice. Panels a, d, e, bar=20 μm; panels b, c, bar=100 μm. No positive signals were detected in the negative control (incubated with only hybridization buffer) (e).

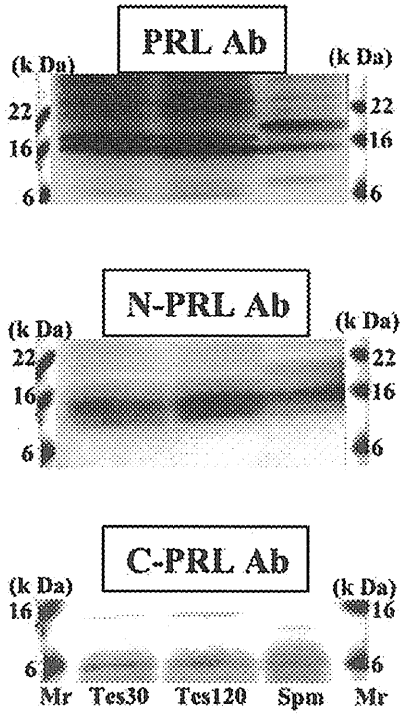

Fig. 3. Immunoblotting of testis and spermatozoa mPRL. Testes and spermatozoa samples resolved by 15% SDS-PAGE were immunoblotted and reacted with N-PRL Ab, C-PRL Ab and PRL Ab. The molecular size of the protein band showed that reactants against N-PRL Ab in the testes were approximately 16-kDa and 17-kDa. In sperm, immunoreactive protein against PRL Ab was approximately 16-kDa and 18-kDa. Full-length PRL is absent in sperm. The size of the protein band indicating binding to C-PRL Ab was less than 6-kDa in both the testis and spermatozoa.

expression. We detected PRL mRNA not only in Leydig cells but also in germ cells, particularly in spermatogonia (Fig. 2B-a), pachytene stage spermatocytes (Fig. 2B-c) and preleptotene stage spermatocytes (Fig. 2B-d) from 10- to 120-day-old mice (Fig. 2B). No signal was detected in the negative control sections that were incubated with only hybridization buffer. Fig. 2-e shows 120 days as a representative sample of the negative control.

Immunoblotting

Immunoblotting also detected mPRL in mature male testes and spermatozoa. The molecular size of the protein band reacted to N-PRL Ab in the testes and spermatozoon was approximately 16 kDa. The size of the protein band reacted to C-PRL Ab was less than 6 kDa in both the testis and spermatozoa. Another faint immunoreactive band was detected by N-PRL Ab and C-PRL Ab immunoblotting, but it could be ignored as the quantity was small. PRL Ab recognized 23-kDa and 16-kDa PRL (Fig. 3). Besides the 16-kDa band, both the PRL Ab and the N-PRL antibodies detected a 17-kDa immunoreactive protein in the testis and an 18-kDa protein in sperm. However, full-length PRL was absent in sperm (Fig.

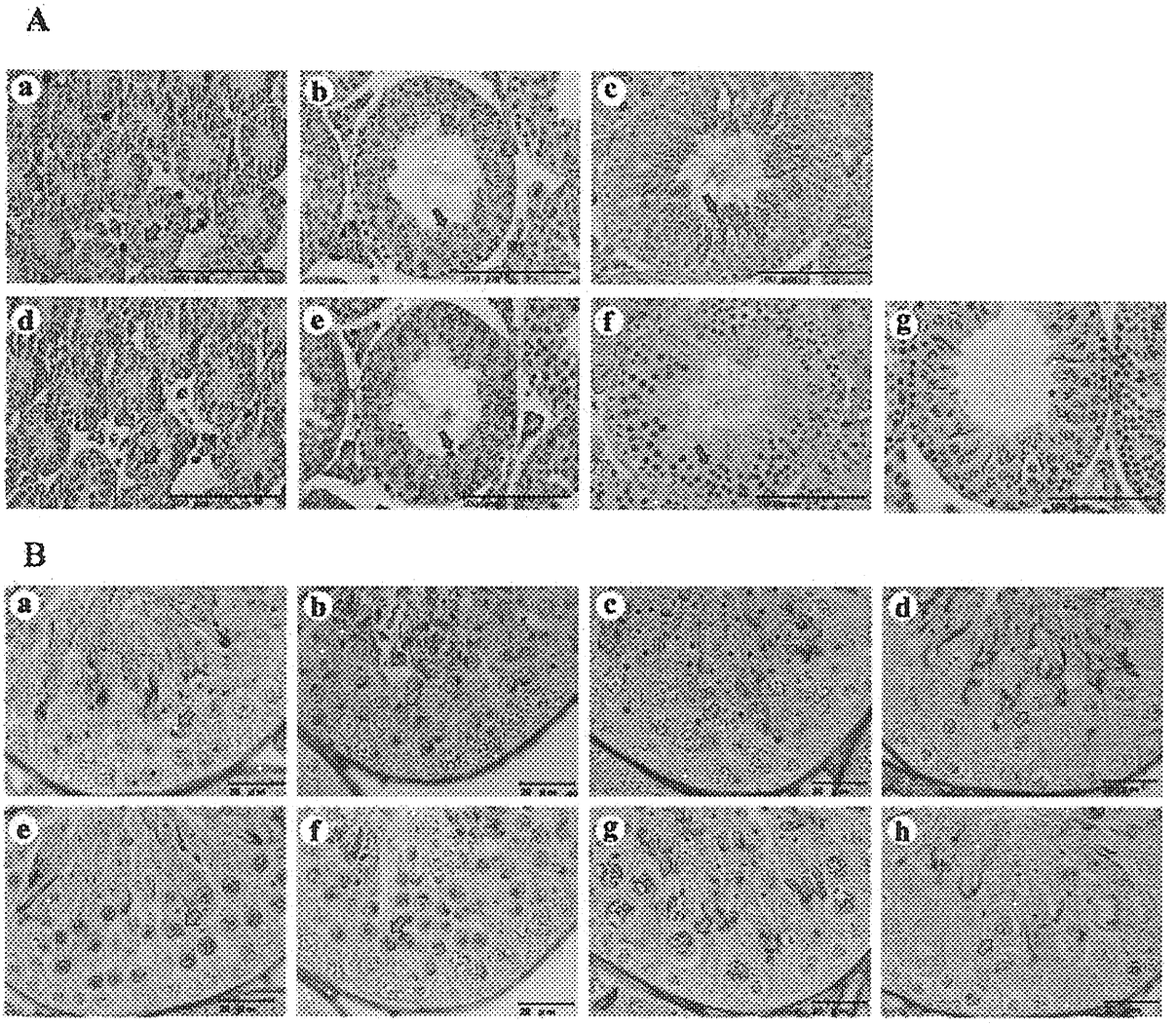

Fig. 4. A: Immunohistochemistry of PRL in testes. The upper panels (a–c) show N-PRL Ab immunoreactivity. The lower panels (d–f) shows C-PRL Ab immunoreactivity. N-PRL Ab and C-PRL Ab immunoreactive sections of testes from days 10 (a, d), 30 (b, e) and 120 (c, f) were the serial sections. Localization of N-PRL Ab staining was clearly different from that of C-PRL Ab. Bar=100 μm. No positive signals were detected in the negative control (incubated with PBS) (g). B: Stage-specific localization of N-terminal cleaved PRL and C-terminal cleaved PRL. The upper panels (a–d) show N-PRL Ab immunoreactivity. The lower panels (e–h) show C-PRL Ab immunoreactivity followed by a PAS reaction for the day 120 testis. N-PRL Ab and C-PRL Ab immunoreactive sections were the serial sections at stages IV (a, e), VI (b, f), IX (c, g) and XII (d, h). Different immunoreactivity was detected in the early round to elongated spermatids. Bar=20 μm.

3). In the day10 testis, faint bands were detected by immunoblotting of each antibody (data not shown). The N-PRL Ab positive band was abolished by absorption with the recombinant mPRL (data not shown).

Localization of PRL in the mouse testis and spermatozoa

For the immunohistochemical studies, the absorption test in which each antibody was preincubated with the recombinant mPRL (1 μg/ml) demonstrates the specificity of the antibodies because no signal was detected.

PRL was detected in the Leydig cells of testes from 10- to 120-day-old mice and in early round and elongated spermatids from testes after day 30 by immunohistochemistry (Fig. 4A). These specific signals were located in different areas compared with PRL mRNA localization. Furthermore, the N-PRL Ab (Fig. 4A-b, c) and C-PRL Ab (Figs. 4A-e, f) immunoreacted signals were not localized in the same area.

Stage-specific localization of PRL in testicular germ cells was

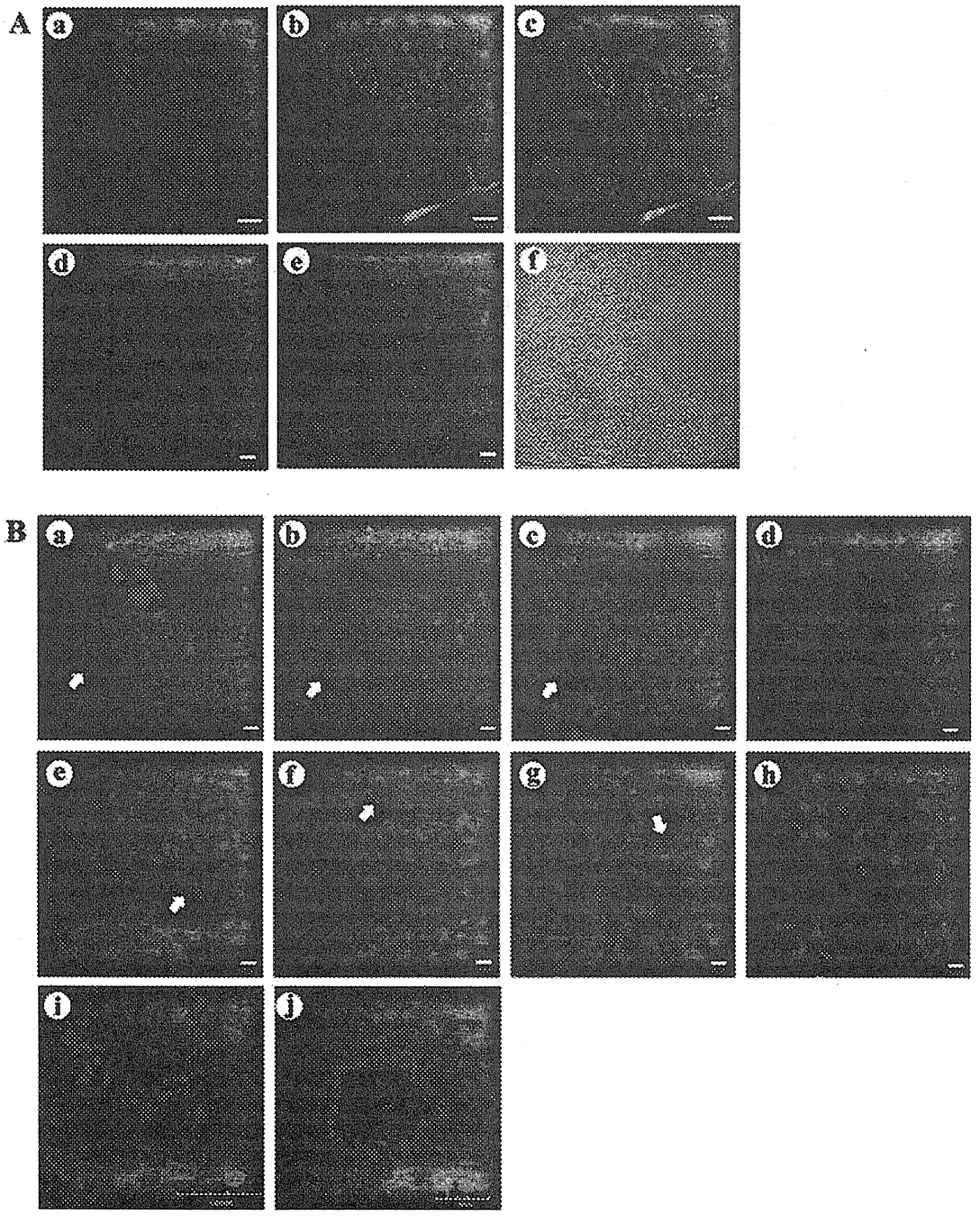

Fig. 5. A: Immunofluorescence double staining for mPRL (red) and Golgi 58K protein (green). Yellow indicates that N-PRL Ab immunoreactivity completely colocalized with Golgi 58K protein (c). Panels d and e show high magnification images of N-PRL Ab (a) and Golgi 58K protein (b). Panel f shows a bright field image of the negative control (incubated with PBS). The nuclei were counterstained with DAPI (blue). Panels a–c, bar=100 μm; panels d–f, bar=10 μm. B: Immunohistochemical staining for mPRL. The PRL signal is shown in red. Panels a–c show immunoreactivity for the testis from day 120. Panel e–g shows immunoreactivity for mature male mouse spermatozoa from the cauda epididymis. Panels a and e show immunoreactivity for N-PRL Ab. Panels b and f show immunoreactivity for C-PRL Ab. Panels c and g show immunoreactivity for PRL Ab. N-PRL Ab staining was localized in elongated spermatid tails from testes and in spermatid tails of spermatozoa. C-PRL Ab staining was localized in the testis, elongated spermatid head pieces and spermatozoa head pieces. PRL Ab staining was localized in both regions. No positive signals were detected in the negative control (incubated with PBS) (d, h). No signal was detected in the absorption test for N-PRL Ab (i) and C-PRL Ab (j). The nuclei were counterstained with DAPI (blue). Bar=10 μm.

detected by immunostaining with N-PRL Ab and C-PRL Ab followed by PAS staining (Fig. 4B). N-PRL Ab reacted with protein in the Golgi-like regions of spermatids, nuclei of round spermatids, cytoplasms of elongated spermatids and in the spermatozoa tails. On the other hand, C-PRL Ab reacted with protein in the nuclei of round spermatids and in the spermatozoa heads. Spermatogonia through mid-pachytene spermatocytes showed no immunoreactivity. PRL was first detected in late pachytene spermatocytes at stage IX. Different immunoreactivity for N-PRL Ab and C-PRL Ab was detected in the early round to elongated spermatids (Fig. 4B).

Because concentrated N-PRL Ab immunoreactivity was observed in a discrete perinuclear region, we then analyzed whether this was in the Golgi apparatus. Double-staining immunohistochemistry was performed with N-PRL Ab and anti-Golgi 58K as the Golgi marker. N-PRL Ab immunoreactivity was partially colocalized with Golgi 58K protein (Fig. 5A).

Counterstaining was performed with DAPI following immunofluorescence staining with N-PRL Ab, C-PRL Ab and PRL Ab in adult testis. The data clearly indicated that immunoreactivity of N-PRL Ab was present in the elongated spermatid tails, while C-PRL Ab immunoreactivity was observed in elongated spermatid head pieces (Figs. 5B a, b). As expected, PRL Ab, which recognizes all PRL forms, reacted with elongated spermatid tails and head pieces that seemed to be in the acrosome region (Fig. 5B c). Interestingly, cleaved PRL variants and intact PRL localized differently. Therefore, we performed immunofluorescence staining with N-PRL Ab, C-PRL Ab and PRL Ab of spermatozoa collected from the cauda epididymides of mature male mice. The immunoreactivity of the male mouse spermatozoa was the same as that of the adult testis; that is, N-PRL Ab reacted with protein in spermatid tails, C-PRL Ab reacted with spermatid head pieces and PRL Ab reacted with both spermatid tails and head pieces (Figs. 5B e–g).

Discussion

The results presented here demonstrate that PRL is ectopically expressed in the testes. Furthermore, PRL in the testes and spermatozoa is cleaved, and cleaved PRL variants are localized in the Golgi apparatus of spermatids and in spermatozoa tails.

Our data also indicate that PRL mRNA is localized not only in Leydig cells but also in germ cells, particularly in the spermatogonia of the testes. The real-time RT-PCR analysis showed that the level of ectopic PRL expression was particularly high in the day 7 testis. In the day 7 testis, there were only spermatogonia to the germ cells, and this was consistent with the *in situ* hybridization signal.

The real-time RT-PCR analysis was performed with primers that span the intron. Furthermore, the testis PRL sequence was the same as a pituitary PRL. Emanuele NV *et al.* [19] previously described a mutant with exon 4 deleted that also encodes a 16-kDa protein in the brain. This mutant was not found in the testis. Although the ectopic PRL function remains unclear, these extrapituitary PRLs are thought to play a role in controlling local functions of the specific tissue, since the amount of PRL produced in extrapituitary tissues is much less than that in the pituitary gland. For instance, it is speculated that decidual PRL regulates uterine water balance or fetal functions that PRL in the immune system stimulates proliferation of lymphocytes and that PRL in the brain modulates a number of behavioral and neuroendocrine controls [17]. On the other hand, male reproductive function is not affected in PRL KO and PRLR KO mice; however, testis PRL may play a role in controlling spermiogenesis and spermatogenesis.

Immunoblotting also detected mPRL in the testes and spermatozoa, but the molecular size of the immunoreactive substance was smaller than that of full-length PRL. Western blot analysis suggests that 16-kDa and 17-kDa immunoreactive proteins exist in the testis. On the other hand, 16-kDa and 18-kDa immunoreactive proteins exist in sperm. Full-length PRL is also absent in sperm. It is suggested that other antiangiogenic PRL fragments were truncated by the testis and sperm. Absence of full-length PRL in sperm suggests that PRL was cleaved at the testes or 23-kDa PRL in the testes.

Based on this finding, we focused on N-terminal cleaved PRL to determine its involvement in spermatogenesis. The N-terminal cleaved PRL variants can be derived from an enzymatically cleaved form of intact PRL, and they have unique functions that are not shared with the parent PRL. The cleaved PRL variants were first reported in rat pituitary extracts by Mittra [20, 21]. Interestingly, the specific function of cleaved PRL variants includes the inhibition of angiogenesis, tumor vascularization and growth and induction of apoptosis as a means of blood vessel regression [22]. Moreover, cleaved PRL variants were recently shown to mediate postpartum cardiomyopathy, a life-threatening disease in late-term and lactating mothers [23, 24].

Based on these reports, we speculate that the functions of cleaved PRL variants are different from those of intact PRL in the male reproductive system.

In situ hybridization revealed that mouse PRL mRNA was expressed in spermatogenic cells. Although strong signals were detected in spermatogonia by *in situ* hybridization, immunohistochemistry showed unexpectedly no reaction in spermatogonia for all antibodies. Whether PRL protein was not translated in spermatogonia or PRL disappeared from these cells remains in question.

Immunohistochemical analysis suggested that full-length PRL is localized in the nuclei of round spermatids, while cleaved PRL variants appear to be localized in the Golgi apparatus of the spermatid nuclei of round spermatids, cytoplasms of elongated spermatids and in the spermatozoa tails.

In contrast to hormones that are endocytosed and then rapidly degraded in lysosome, plasma-borne PRL is transported by transcytosis across the lactating mammary epithelial cell through the endocytotic and secretory pathways and released in milk in an intact molecular form of intact and cleaved molecular forms [9]. PRL is transported via a vesicular pathway that includes not only endosomes, late endosomes and multivesicular bodies, but also vesicles located in the Golgi region and secretory vesicles containing casein micelles [25]. There may be a similar mechanism at work in the testes and spermatozoa that is similar to that in the mammary glands. However, PRL does not appear to be released extracellularly in the testes and spermatozoa or to accumulate in spermatozoa tails and heads. Whether cleaved PRL variants exert

their effects through a classical transmembrane receptor remains to be determined. Cleaved PRL variants derived from PRL bind to a single class of sites on endothelial cell membranes with a Kd of 1 - 10 nM, and cross-linking experiments have identified proteins of 52-kDa and 32-kDa as the major cleaved PRL variant binding species [11]. It is still unclear whether these proteins are receptors for cleaved PRL variants or regulatory binding proteins important for cleaved PRL function.

The present study suggests that PRL originating the testes and cleaved PRL variants may play a role in spermiogenesis, and spermatogenesis and that the signal transduction and secretory mechanisms at work may differ from those of intact PRL.

Acknowledgments

This research was supported by funding for the "High-Tech Research Center" Project for Private Universities and a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2006–2008).

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