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Authors: Kido, Tatsuo, and Namiki, Hideo

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Evidence that the PERF 15 Germ Cell Specific Protein Associates with DNA in the Presence of Ca^{2+}

Tatsuo Kido and Hideo Namiki*

Department of Biology, School of Education, Waseda University, Shinjuku-ku, Tokyo 169-8050, Japan

ABSTRACT—Metallic ion-mediated interactions between chromosomal proteins and DNA during mammalian spermatogenesis are poorly understood. In search of specific proteins related to this process, we fractionated soluble proteins from rat testis using Ca^{2+} and DNA in low ionic strength solution. A testis specific 16 kDa protein that sedimented in the presence of both Ca^{2+} and DNA was identified as PERF 15 (Swiss-Prot ID: TLBP_RAT) based on amino acid sequence and immunochemical analysis. PERF 15 is the major germ cell protein of the rat perforatorium, but its role during spermatogenesis is unknown. Indirect immunofluorescence showed that PERF 15 was present in the nucleus from spermatocyte to middle spermatid stage. In late-pachytene spermatocytes, PERF 15 colocalized with DNA. In addition, PERF 15 contained phosphotyrosine like other fatty acid binding proteins with high similarity. These results suggest that PERF 15 specifically associates with germ cell chromatin and that its function may be regulated by Ca^{2+} and tyrosine phosphorylation.

INTRODUCTION

During mammalian spermatogenesis, germ cell chromatin undergoes dramatic changes in transcriptional activity (Kierszenbaum and Tres, 1975) while its structure is transformed into that of mature sperm (Grimes and Smart, 1985). Specific nucleoproteins are translated at each step of spermatogenesis, and these are thought to be associated with progressive alterations in the structural organization of chromatin (Mayer and Zirkin, 1979). Recently, several such proteins have been identified and characterized (Seyedin and Kistler, 1980; Moss *et al.*, 1989; Kistler *et al.*, 1996). In addition, Ca^{2+} has been localized in the nucleus of rat spermatogenic cells, and a role for this ion in cell growth and differentiation has been suggested (Ravindranath *et al.*, 1994). Indeed, the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, was identified as an enzyme responsible for remodeling of nuclear chromatin in spermatids (Moriya *et al.*, 1995). Nevertheless, there are few reports about the interaction between Ca^{2+} and chromatin structures during spermatogenesis.

Therefore, we biochemically fractionated testicular proteins. Here we report on the identification and characterization of the germ cell specific 16 kDa protein fractionated as a protein which had Ca^{2+}-dependent association with DNA.

* Corresponding author: Tel. +81-3-5286-1507; FAX. +81-3-3207-9694.

MATERIALS AND METHODS

Fractionation

Various organs, brain, heart, lung, liver, kidney, spleen, thigh muscle, and testis of adult Wistar-Imamichi rats were homogenized in 3 volumes of ice cold 50 mM Tris-HCl buffer (pH 7.2) containing 25 mM KCl and 5 mM $MgCl_2$ (TKM solution). After centrifugation at 1,500 ×g for 30 min, pellets were resuspended in an equal volume of 50 mM Tris-HCl buffer (pH 7.2) containing 0.8 M NaCl and incubated for 30 min at 37°C. After centrifugation at 40,000×g for 1 hr, the supernatant was dialyzed against 20 mM Tris-HCl buffer (pH 7.2) overnight at 4°C. Soluble proteins were collected in supernatant (A) following centrifugation at 100,000×g for 1 hr to remove insoluble proteins that remained after dialysis. Protein concentrations were measured by dye-binding assay (Bio-Rad protein assay kit, Bio-Rad Laboratories, USA) using bovine serum albumin (BSA) as a standard. Soluble proteins were fractionated (Fig. 1) after equalizing the protein concentration to 2.5 mg/ml between each organ. Supernatant (A) was made 10 mM in $CaCl_2$, and the solution was incubated for 30 min at 37°C and centrifuged at 100,000×g for 1 hr. The resultant supernatant was designated supernatant (B), and the pellet was designated pellet (C). Supernatant (B) was made 12 mM in EDTA and incubated. After centrifugation, the resultant supernatant (D) was added 0.4 mg/ml highly purified calf thymus DNA (type I, SIGMA, USA), incubated, and centrifuged again. Finally, supernatant (F) was added 12 mM $CaCl_2$ and another 0.4 mg/ml DNA, and the solution was incubated and centrifuged. The resultant pellet, sedimented in the presence of both Ca^{2+} and DNA, was designated pellet (I).

Experiments above mentioned was also repeated in the homologous system utilizing rat DNA extracted from rat liver by the procedure of Sambrook *et al.* (1989).

Electrophoresis and electrotransfer

SDS-PAGE was performed on a 16% acrylamide gel (Laemmli, 1970). Two-dimensional PAGE was performed under non-denaturing conditions by modification of the procedure of Manabe *et al.* (1979).

Isoelectric focusing (IEF) with ampholytes at final concentrations of 5% (Bio-Rad Laboratories, USA) were carried out at 100V for 1 hr and then at 200 V for 4 hr at 4°C. Gels were equilibrated in Laemmli sample buffer for 20 min and then loaded onto 16% acrylamide gels. The second run was carried out at 20 mA. Rectangular gels (2 mm× 80 mm) (ATTO, Japan) were used for IEF-PAGE, and mini-gels (90 mm×80 mm) (ATTO, Japan) were used for SDS-PAGE. Gels were either stained using a Silver Stain Kit *Wako* (Wako Pure Chemical Industries, Ltd., Japan), or proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) by the semi-dry procedure (Kyhse-Andersen, 1984).

Peptide sequencing

Pellet (I) from testis was resuspended in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to a PVDF membrane (Applied Biosystems, USA). After staining the membrane with Coomassie Brilliant Blue R250 (MERCK, Germany), the 16 kDa protein band was cut out and analyzed on a protein peptide sequencer (477A, Applied Biosystems, USA). The obtained sequence was compared with the Swiss-Prot database through FASTA program (Pearson and Lipman, 1988) on GenomeNet server. The Swiss-Prot database consists of protein sequences (a joint collaboration between the University of Geneva and European Bioinfomatics Institute, Cambridge, Cambs, UK).

Antibodies

Anti-PERF 15[40–59] was a rabbit polyclonal antiserum raised against the synthetic peptide, PERF 15[Ser^{40}-Arg^{59}]. The peptide was conjugated with Keyhole Limpet Hemocyanin (KLH) and emulsified with Freund's complete adjuvant (Wako Pure Chemical Industries, Ltd., Japan). Anti-phosphotyrosine (PY20) was a mouse monoclonal antibody (Transduction Laboratories, USA).

Immunoblots

Proteins transferred to PVDF membranes were probed with either anti-PERF 15[40–59] or PY20 at a dilution 1:100. Immunoreactive proteins were detected using a VECTASTAIN ABC KIT (Vector Laboratories, Inc., USA).

DNA-cellulose binding assay

DNA-cellulose binding assay was performed by modification of the procedure of Bouhoute and Leclercq (1995). The incubation mixture contained 100 µl DNA-cellulose (double-stranded prepared from calf thymus DNA, SIGMA, USA) corresponding to 175 µg DNA, 500 µl supernatant (A) and either 0 or 1 mM $CaCl_2$. The mixture was incubated for 30 min at 37°C. After incubation, the DNA-cellulose was pelleted by centrifugation at 2,000×g for 1 min and washed twice with 50 mM Tris-HCl buffer (pH 7.2) containing either 0 or 1 mM $CaCl_2$, respectively. The pellet was extracted for 1 hr at 37°C with 50 µl Laemmli sample buffer. Parallel assays using cellulose (microgranular, SIGMA, USA) in place of DNA-cellulose gave the level of non-specific binding.

Indirect immunofluorescence

Testes of adult Wistar-Imamichi rats were cut into 5 mm×5 mm pieces and fixed in Bouin's solution for 2 days. Fixed tissue was washed several times in 70% ethanol and processed for paraffin embedding. Sections were cut at 5 µm and were mounted on glass slides. Sections were deparaffinized in xylene, and picric acid was removed by washing in 96% ethanol for 1 day. After hydration in a graded ethanol series, slides were washed in PBS. To reduce non-specific background, sections were treated with 5% BSA in PBS for 1hr at room temperature. They were then incubated with a 1:100 dilution of either anti-PERF 15[40–59] or preimmune serum for 1 hr at 37°C, washed with PBS, incubated with a 1:100 dilution of rhodamine-conjugated anti-rabbit IgG goat antibody (Organon Teknika Corp., USA) containing 0.1 µg/ml 4', 6-Diamidino-2-phenylindole Dihydrochloride (DAPI) (Wako Pure Chemical Industries, Ltd., Japan) for 1 hr at 37°C, and washed again with PBS. Sections were mounted with coverslips and examined by fluorescence microscopy (ECLIPSE E600, Nikon, Japan).

RESULTS

Protein fractionation

In non-denaturing low ionic strength solution, short lived DNA-proteins complexes such as histones H2A and H2B were well-defined and sedimented by centrifugation (Aragay *et al.*, 1988). In the present study, we solubilized proteins from various organs in high ionic strength solution (0.4 M NaCl), and fractionated them at low ionic strength using Ca^{2+} and DNA (Fig.1) by employing a novel method. Proteins, whose sedimentation depended on either Ca^{2+} or DNA, were distributed and removed into pellets (C), (E), and (G). In pellet (I), proteins whose sedimentation depended on both Ca^{2+} and DNA were collected. A prominent 16 kDa protein was detected in pellet (I) from testis (arrow in Fig. 2a). Using rat liver DNA instead of calf thymus DNA in the fractionation, the 16 kDa protein was also detected prominently in pellet (I) (allow in Fig. 2b). The 16 kDa protein was detected prominently in the EGTA solubilized proteins from pellet (I) sedimented using Ca^{2+} (lane Ca in Fig. 2c). However, when Mg^{2+} was used in place of Ca^{2+}, the 16 kDa band was detected unclearly in the EDTA solubilized proteins (lane Mg in Fig. 2c). These results suggest that the 16 kDa protein may associate with DNA in the presence of Ca^{2+}.

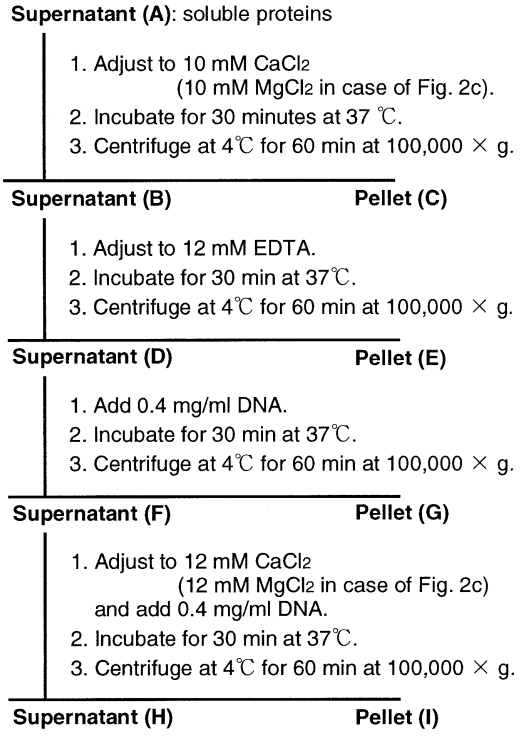

Fig. 1. Flow chart of fractionation.

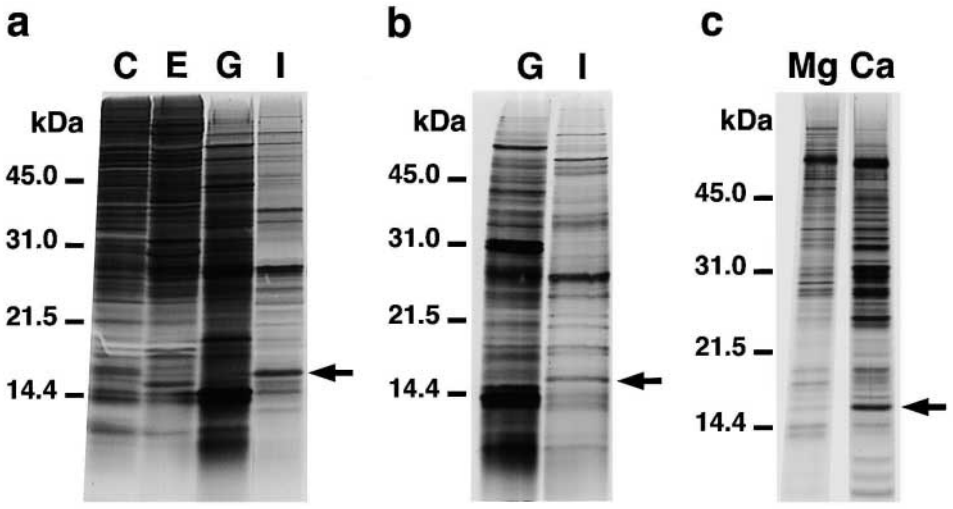

Fig. 2. SDS-PAGE analysis of each fraction of testis soluble proteins. In the experiment **a** and **b**, each pellet was resuspended in sample buffer. In the experiment **c**, each pellet was resuspended in 20 mM Tris-HCl buffer (pH 7.2) containing either 25 mM EDTA (lane Mg) or 25 mM EGTA (lane Ca). Samples were subjected to SDS-PAGE followed by silver stain. (**a**) Each pellet sedimented using calf thymus DNA and Ca^{2+} as described in materials and methods. 50 μg of soluble proteins (contained in supernatant (A)) were fractionated according to the flow chart (Fig. 1). Sample letters are keyed in Fig. 1. (**b**) Samples sedimented using rat liver DNA instead of calf thymus DNA. 60 μg of soluble proteins were fractionated. (**c**) Comparative study of pellet (I) sedimented using either Ca^{2+} (lane Ca) or Mg^{2+} (lane Mg). 500 μg of soluble proteins were fractionated. The 16 kDa band indicated by the arrows.

Moreover, since comparison with other organs showed that the 16 kDa protein was testis specific (Fig. 3), the 16 kDa protein probably have a role in male reproductive system.

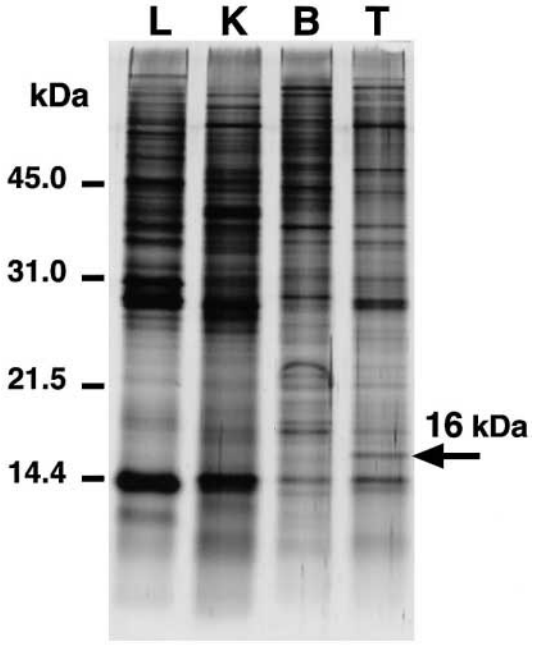

Fig. 3. SDS-PAGE analysis of proteins in pellet (I) from various organs. Pellet (I) from liver, kidney, brain, and testis were resuspended in sample buffer and were subjected to SDS-PAGE followed by silver stain. 150 μg of soluble proteins of each organ were fractionated. Lane L, liver; lane K, kidney; lane B, brain; lane T, testis. Note that the 16 kDa protein is detected only in testis (arrow).

Identification of 16 kDa protein

To identify the 16 kDa protein, we determined the N-terminal 27 amino acids sequence as described in Materials and Methods. The sequence was 'MIEPFLGTWKLVSSENFENYVRELGVE', identical to PERF 15 (ID: TLBP_RAT) extracted from the Swiss-Prot database. To detect PERF 15 specifically, we synthesized a peptide corresponding to a PERF 15 specific region, Ser^{40}-Arg^{59} (underline in Fig. 4), and raised a rabbit polyclonal antibody against the peptide conjugated with KLH (Fig. 5a). Immunoblots of soluble proteins from various organs showed only a single immunoreactive 16 kDa band in testis (Fig. 5b), evidence that anti-PERF 15[40–59] specifically recognized PERF 15. Next, we determined whether the 16 kDa protein in testis pellet (I) was PERF 15. Proteins in supernatant (A) and each pellets from testis were resolved on SDS-PAGE and immunoblotted with anti-PERF 15[40–59]. An immunoreactive 16 kDa band was detected only in supernatant (A) and pellet (I) (Fig. 6). These results are consistent with the idea that the 16 kDa protein is PERF 15.

DNA-cellulose binding assay

The interaction between PERF 15 and DNA was confirmed by DNA-cellulose binding assay. In the previous studies of calmodulin, the protein-protein interactions were analyzed in the presence of 1–2 mM Ca^{2+} (Moriya *et al.*, 1993; Trejo and Delhumeau, 1997). Therefore, DNA-cellulose binding assay was also performed at a concentration of either 0 or 1 mM Ca^{2+}. The PERF 15 retained on DNA-cellulose or DNA-free cellulose was immunodetected with anti-PERF 15[40–59]. PERF 15 retained weakly on the DNA-free cellulose either in the presence of Ca^{2+} or not (Fig. 7, lanes 1 and 2), and the retention of PERF 15 was regarded as non-specific bind-

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                1           11          21          31          41          51          61          71
PERF 15  :  MIEPFLGTWK LVSSENFENY VRELGVECEP RKVACLI  KPS VSISFNGERM DIQAGSACRN TEISFKLGEE FEETTADNRK
MYP2     :  -SNK------ -----H-DD- MKA---GLAN --LGN-A  --T -I--KK-DYI T-RTE--FK- --------Q- -D--------
H-FABP   :  -ADA-V---- --D-K--DD- MKS---GFAT -Q--SMT  --T TI-EK--DTI T-KTH-TFK- -----Q--V- -D-V---D--
ALBP     :  -CDA-VG--- -------DD- MK-V--GFAT ----GMA  --N MI--V--DLV T-RSE-TFK- --------V- -D-I---D--
CRABP    :    PN-A---- MR-----DEL LKA---NAML ----VAAAS--H -E-RQD-DQF Y-KTSTTV-T ---N--V--G ---E-V-G--

                81          91             101         111         121         131
                VKSLITFEG G SMIQIQRWL   G KQTTIKRRIV DGRMVVECTM NNVVSTRTYE RV
                A--IV-L-R - -LK-V-K-D   - -E-A-R-TLL --------I- KG--C--I-- K-
                ---VV-LD- - KLVHV-K-D   - QE--LT-ELS --KLILTL-H G--------- KEA
                ---I--LD- - ALV-V-K-D   - -S-----KRD GDKL----V- KG-T---V-- -A
                CR--P-W-NEN KIHCT-TL-EGD- PK-YWT-ELA NDELILTFGA DD--C--I-V -E
```

Fig. 4. Amino acid sequences of fatty acid binding proteins with high similarity to PERF 15. PERF 15, rat PERF 15 (Oko and Morales, 1994); MYP2, mouse myelin P2 protein (Narayanan *et al.*, 1991); H-FABP, mouse heart fatty acid binding protein (Heuckeroth *et al.*, 1987); ALBP, mouse adipocyte lipid binding protein (Matarese and Bernlohr, 1988); CRABP, mouse cellular retinoic acid binding protein (Rajan *et al.*, 1991). Hyphens mark amino acids identical to PERF 15. The region underlined in PERF 15 shows low similarity with the other proteins.

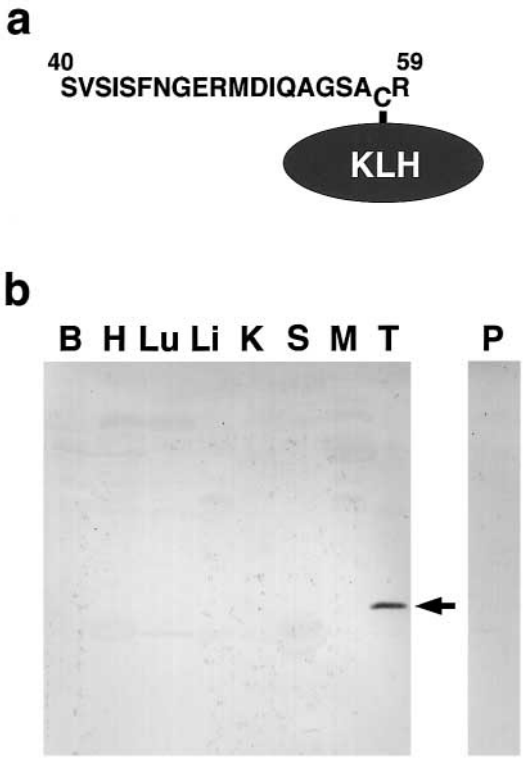

Fig. 5. (**a**) Synthetic peptide antigen. Peptide representing the PERF 15 specific region, Ser[40]-Arg[59], was conjugated with KLH on Cys[58]. (**b**) Immunoreactivity of anti-PERF 15[40-59] on an immunoblot of supernatant (A) from various organs. Samples (50 μg of protein) were subjected to SDS-PAGE, and proteins were immunoblotted using anti-PERF 15[40–59] or preimmune serum. Lanes B-T, probed with anti-PERF 15[40–59]; Lane P, probed with preimmune serum. Lane B, brain; lane H, heart; lane Lu, lung; lane Li, liver; lane K, kidney; lane S, spleen; lane M, thigh muscle; lanes T and P, testis. Note that anti-PERF 15[40–59] recognized the 16 kDa protein only in testis.

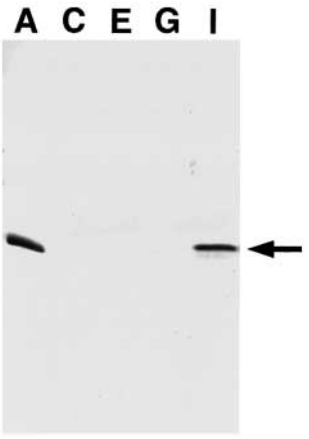

Fig. 6. Immunoblots of each fraction of soluble proteins from testis. Supernatant (A) (50 μg of protein) and each pellet prepared as described in Fig. 2a were subjected to SDS-PAGE and immunoblotted with anti-PERF 15[40–59]. Lanes A-I, sample letters are identical to Fig. 1. Note that PEFR 15 was predominantly detected in pellet (I).

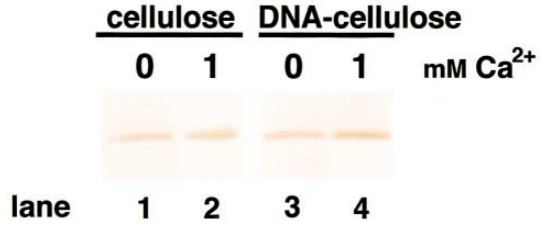

Fig. 7. Binding ability of PERF 15 to DNA-cellulose. Supernatant (A) was incubated with DNA-cellulose or cellulose at the final concentrations of $CaCl_2$ as indicated. Aliquots of each extraction (20 μl) was subjected to SDS-PAGE and immunoblotted with anti-PERF 15[40-59]. Note that PERF 15 was retained strongly on DNA-cellulose in the presence of Ca^{2+} (lane 4).

ing to cellulose. Without Ca^{2+}, the retention of PERF 15 on DNA-cellulose was the same as non-specific binding (Fig. 7, lane 3). In contrast, PERF 15 retained strongly on the DNA-cellulose in the presence of Ca^{2+} (Fig. 7, lane 4). These results support the presumption that PERF 15 associate with DNA depending on Ca^{2+}. However, since PERF 15 has no known DNA-binding sequence (data not shown) and there still co-exist some other proteins in the preparation of PERF 15 (Fig. 2), PERF 15 might associate with another protein and indirectly bind to DNA.

Indirect immunofluorescence

To confirm the interaction between PERF 15 and DNA *in vivo*, we localized PERF 15 and DNA in the sections of testis. Double labelling was performed with rhodamine-conjugated anti-rabbit secondary antibody for anti-PERF 15[40–59], and DAPI for DNA. In mid-pachytene spermatocytes, PERF 15 immunoreactivity was predominantly detected in the cytoplasm, and there was little or none in the nucleus (arrowhead in Fig. 8c). PERF 15 immunoreactivity was appeared in nuclei at the late-pachytene spermatocyte to middle spermatid stages (arrows in Fig. 8a, 8c and arrowheads in Fig. 8e). However, nuclei at late-elongated spermatid stages showed no anti-PERF 15 immunofluorescence (small arrow in Fig. 8a). These observations are consistent with previous reports (Oko and Clermont, 1991; Oko and Morales, 1994; Oko, 1995). In the present study, at the late-pachytene spermatocyte stage, colocalization of PERF 15 with DNA was clearly evident. Although there were some low intensity rhodamine fluorescence as nonspecific background levels in the nucleus, significant fluorescence of immunoreactive PERF 15 that overlapped with DNA were detected (arrowheads in Fig. 8e, f, g). These results suggest that PERF 15 associates with DNA in spermatogenical cells for a prolonged period during spermatogenesis.

Interestingly, PERF 15 immunoreactivity was observed only in the nucleus at the early stages of spermatocyte (data not shown), but the reason for this remains obscure.

Analysis of PERF 15 phosphorylation

Tyrosine phosphorylation appears to regulate the function of some fatty acid binding proteins (Buelt *et al.*, 1991; Nielsen and Spener, 1993). Therefore, we analyzed whether any of the tyrosines of PERF 15 were phosphorylated using anti-phosphotyrosine antibody PY20. The position of PERF 15 on two-dimensional PAGE was identified initially by immunoblotting with anti-PERF 15[40–59]. Three major im-

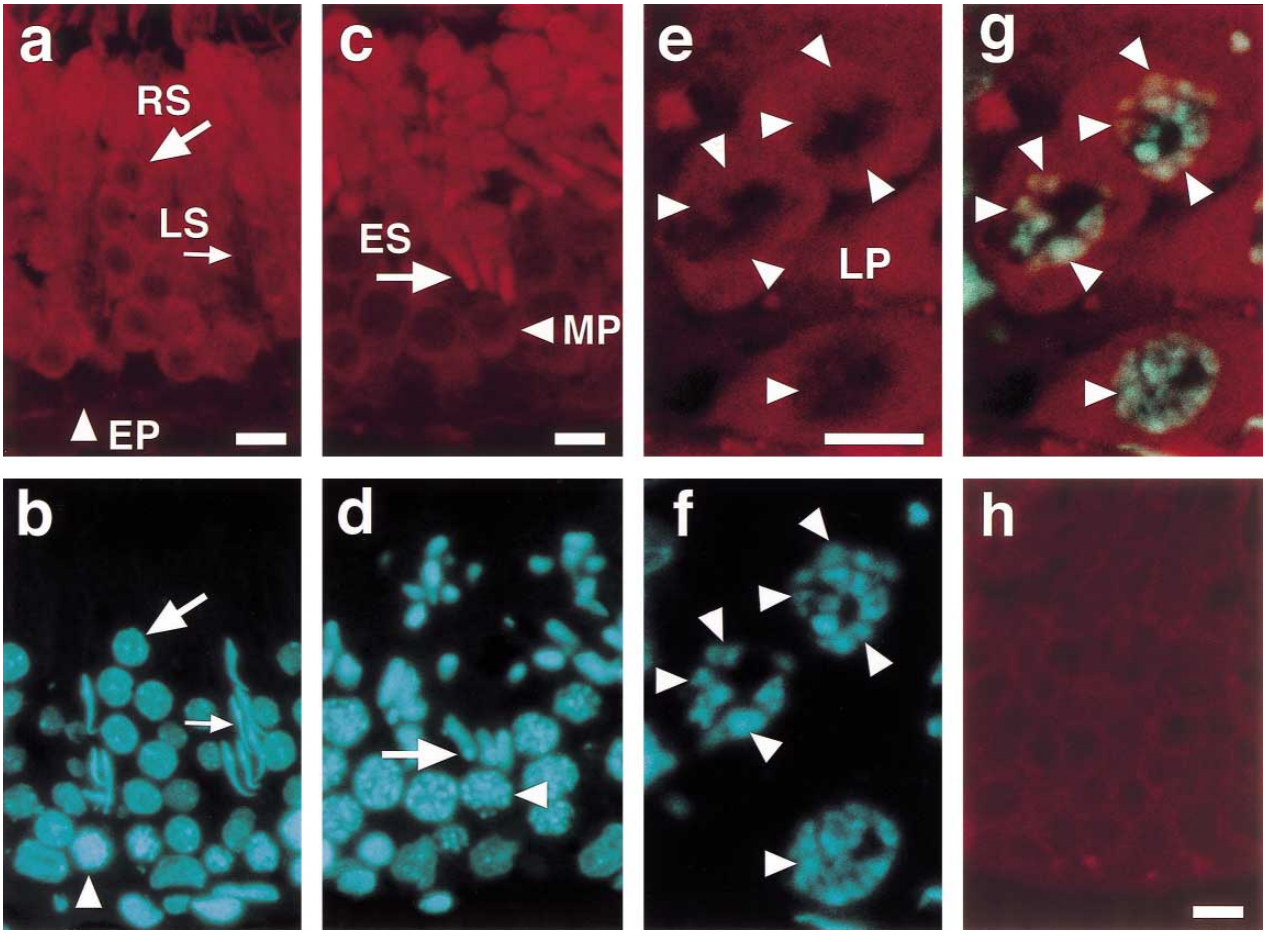

Fig. 8. Immunohistochemistry of testis stained with anti-PERF 15[40-59]. Immunoreactivity was detected with rhodamine-conjugated goat anti-rabbit IgG using fluorescence microscopy (**a**, **c** and **e**). **b**, **d** and **f** represent the same field stained for DNA with DAPI as **a**, **c** and **e**, respectively. **g** shows the digital combined image of **e** and **f**. EP, early-pachytene spermatocytes; MP, mid-pachytene spermatocytes; LP, late-pachytene spermatocytes; RS, round spermatids; ES, early-elongated spermatids; LS, late-elongated spermatids. Scale bar = 10 μm. Immunoreactive PERF 15 was prominent in the nucleus at stages from the late-pachytene spermatocyte to the early-elongated spermatid stages. Nucleus of late-pachytene spermatocytes show reactivity as spots. These corresponded to DNA densities revealed by DAPI staining of the same field (arrowheads in **e**, **f**, **g**). **h** represents a section stained with preimmune serum as a negative control.

munoreactive spots at pI=4.9, 5.5, 6.2 as well as some minor spots were detected (Fig. 9a). Immunoblots with PY20 showed that a large number of testis proteins, including some of the PERF 15 proteins, contained phosphotyrosine (arrowheads in Fig. 9b). However, the site of PERF 15 phosphorylation could not be determined.

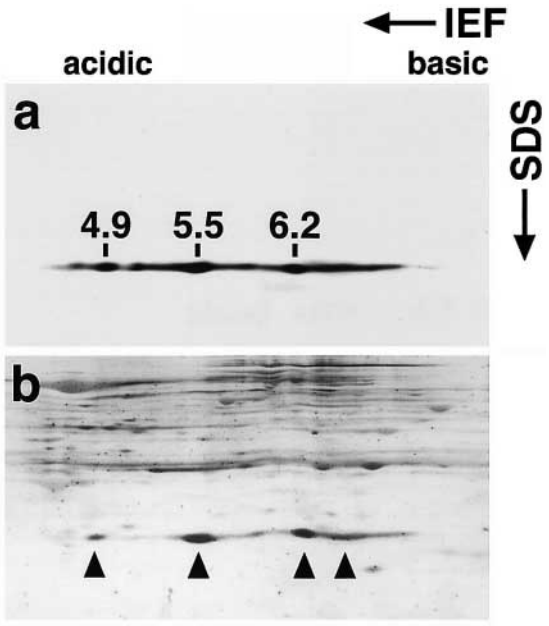

Fig. 9. Testis soluble proteins (150 μg) from supernatant (A) were resolved by two-dimensional gel electrophoresis and transferred to a PVDF membrane. (**a**) Immunoblot with anti-PERF 15[40–59]. Three major immunoreactive spots were detected at pI=4.9, 5.5, and 6.2. Some minor spots also were detected. (**b**) Immunoblot with PY20. A large number of polypeptides were reactive including PERF 15 (arrowheads). Some PERF 15 minor spots in (**a**) were not reactive with PY20.

DISCUSSION

To further clarify the interaction between Ca^{2+} and chromatin structure in mammal spermatogenic cells, we searched for proteins that associate with DNA in the presence of Ca^{2+}. To do so, we solubilized proteins from various rat organs in high ionic strength solution, and fractionated the soluble proteins using Ca^{2+} and DNA in low ionic strength solution (Fig. 1). A testis specific 16 kDa protein, whose sedimentation depended on both Ca^{2+} and DNA, was found in pellet (I) (Fig. 2, 3). Conceivably, the proteins detected in pellet (I) may associate directly or indirectly with DNA in the presence of Ca^{2+}. Moreover, the 16 kDa protein was not detected in 18 days old rats testis (data not shown). These results suggest that this protein may have a specific role in nuclei during spermatogenesis.

Amino acid sequence analysis showed the N-terminal 27 amino acids sequence of the 16 kDa protein was identical with that of PERF 15. PERF 15 is the major protein of the rat perforatorium found between the inner acrosomal and the outer face of the nuclear envelope of the sperm head. PERF 15 belongs to a family of fatty acid binding proteins and is very similar to myelin P2, adipocyte lipid binding protein (ALBP), and heart fatty acid binding protein (H-FABP) (Oko and Morales, 1994) (Fig. 4). To identify the 16 kDa protein, we raised an antiserum, which specifically recognized PERF 15 and distinguished it from other fatty acid binding proteins. The polypeptide sequence of PERF 15 extending from Ser^{40} to Arg^{59} shows low similarity to others (underline in Fig. 4) and the antiserum raised against this synthetic peptide recognized only PERF 15 on immunoblots (Fig. 5b). When each fraction of testis was analyzed by immunoblotting with anti-PERF 15[40–59], PERF 15 was detected only in pellet (I) (Fig. 6). These results suggest that the 16 kDa protein is PERF 15.

PERF 15 was presumed to be required for attachment of the acrosome to the sperm nucleus during spermatogenesis and fertilization (Oko and Morales, 1994; Sutovsky *et al.*, 1997). The sequence of the gene encoding PERF 15 has been reported recently (Pouresmaeili *et al.*, 1997). Schmitt *et al.* (1994) analyzed immunoreactivity with anti-cellular retinoic acid binding protein (CRABP) antibody and peptide sequence structure of PERF 15, suggesting it may share physicochemical characteristics with CRABP.

In the current study, because PERF 15 was retained strongly on the DNA-cellulose in the presence of Ca^{2+} (Fig. 7), it was presumed that PERF 15 associated with chromatin structures. While PERF 15 appears to accumulate in the nucleus at the spermatocyte to mid-spermatid stages, its biochemical characteristics and biological functions in nuclei are unknown (Oko and Clermont, 1991; Oko, 1995). Accordingly, we examined whether PERF 15 colocalized with DNA during spermatogenesis by indirect immunofluorescence. In early-elongated spermatids, PERF 15 immunoreactivity was uniformly distributed in the nucleus (arrow in Fig. 8c). In elongating spermatids undergoing chromatin condensation, calcium ions were detected previously as large nuclear deposits (Ravindranath *et al.*, 1994). Regarding the role of Ca^{2+} at this stage, it has been hypothesized that Ca^{2+} activates calcineurin through calmodulin, resulting in dephosphorylation of transitional proteins or protamines and condensation of chromatin in maturing sperm heads (Moriya *et al.*, 1995). The presence of PERF 15 in the nucleus of elongated spermatids together with its Ca^{2+}-dependent association with DNA suggests that PERF 15 may participate in chromatin condensation.

At the late-pachytene to secondary spermatocyte stages, when DNA is localized as a discrete chromosomal body, nuclear PERF 15 was detected only in the region known to contain DNA (arrowheads in Fig. 8e, f, g). These observations support the hypothesis that PERF 15 associates with DNA either directly or indirectly *in vivo* and further suggest that PERF 15 might be required for the stage-specific changes in chromatin structure during spermatogenesis.

We also analyzed direct Ca^{2+} binding to PERF 15 using $^{45}Ca^{2+}$, but no such binding was evident (data not shown). Hogan *et al.* (1986) suggested that the structural changes in

DNA caused by Ca^{2+} might regulate the interaction between proteins and DNA. The interaction between PERF 15 and DNA may be regulated to be the same, too. While, since PERF 15 contains no known DNA binding motif (analyzed by PSORTII (Nakai and Kanehisa, 1992) on GenomeNet WWW server), it is possible that PERF 15 associates with DNA via intermediate proteins that require Ca^{2+}.

Myelin P2, ALBP, and H-FABP functions are considered to be regulated by tyrosine phosphorylation on Tyr^{20} in response to insulin (Buelt *et al*., 1991; Nielsen and Spener, 1993). For example, Buelt (1992) suggested that phosphorylation decreases ALBP fatty acid binding activity. PERF 15 may be also regulated by tyrosine phosphorylation. Therefore, we analyzed tyrosine phosphorylation of PERF 15 on immunoblots probed with anti-phosphotyrosine antibody, and the results showed that PERF 15 was phosphorylated on tyrosine (Fig. 9a, b). However, while myelin P2, ALBP, and H-FABP contain a recognition sequence for insulin receptor kinase, Asn-Phe-Asp-Asp-Tyr^{20} (Pearson and Kemp, 1991; Nielsen and Spener, 1993), PERF 15 does not contain this sequence. Accordingly, PERF 15 may be regulated by another kinase.

Several species of immunoreactive PERF 15 were separated on two-dimensional PAGE (Fig. 9a). Nielsen *et al*. (1990) demonstrated that the binding of fatty acid caused H-FABP to shift pI under native conditions. Whether PERF 15 binds a fatty acid, an acidic retinoid, or another lipid is as yet unknown (Schmitt *et al*., 1994), but it is possible that some of the species representing PERF 15 may result from lipid binding. In addition, PERF 15 may be phosphorylated on various non-tyrosine sites, because PERF 15 contains protein kinase C phosphorylation sites on the positions 8–10, 64–66, 104–106, and 125–127 (analyzed by MOTIF (Ogiwara *et al*., 1996) on GenomeNet WWW Server). Further analyses of phosphorylation and possible ligand binding of PERF 15 are needed to clarify these points.

In this study, we suggest that PERF 15 associates with chromatin structure in the presence of Ca^{2+} in spermatogenic cells and that this association is regulated by phosphorylation. Evidence suggests that PERF 15 may associate with DNA and Ca^{2+} indirectly. Identification of proteins that interact with PERF 15 will be necessary to define the role of PERF 15 during spermatogenesis.

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REFERENCES

Aragay AM, Diaz P, Daban JR (1988) Association of nucleosome core particle DNA with different histone oligomers. Transfer of histones between DNA-(H2A,H2B) and DNA-(H3,H4) complexes. J Mol Biol 204: 141–154

Bouhoute A, Leclercq G (1995) Modulation of estradiol and DNA binding to estrogen receptor upon association with calmodulin. Biochem Biophys Res Commun 208: 748–755

Buelt MK, Shekels LL, Jarvis BW, Bernlohr DA (1991) *In vitro* phosphorylation of the adipocyte lipid-binding protein (p15) by the insulin receptor. Effects of fatty acid on receptor kinase and substrate phosphorylation. J Biol Chem 266: 12266–12271

Buelt MK, Xu Z, Banaszak LJ, Bernlohr DA (1992) Structural and functional characterization of the phosphorylated adipocyte lipid-binding protein (pp15). Biochemistry 31: 3493–3499

Grimes SR Jr, Smart PG (1985) Changes in the structural organization of chromatin during spermatogenesis in the rat. Biochim Biophys Acta 824: 128–139

Heuckeroth RO, Birkenmeier EH, Levin MS, Gordon JI (1987) Analysis of the tissue-specific expression, developmental regulation, and linkage relationships of a rodent gene encoding heart fatty acid binding protein. J Biol Chem 262: 9709–9717

Hogan ME, Hayes B, Wang NC, Austin RH (1986) Ion-induced DNA structure change in nucleosomes. Biochemistry 25: 5070–5082

Kierszenbaum AL, Tres LL (1975) Structural and transcriptional features of the mouse spermatid genome. J Cell Biol 65: 258–270

Kistler WS, Henriksen K, Mali P, Parvinen M (1996) Sequential expression of nucleoproteins during rat spermiogenesis. Exp Cell Res 225: 374–381

Kyhse-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 10: 203–209

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Manabe T, Tachi K, Kojima K, Okuyama T (1979) Two-dimensional electrophoresis of plasma proteins without denaturing agents. J Biochem 85: 649–659

Matarese V, Bernlohr DA (1988) Purification of murine adipocyte lipid-binding protein. Characterization as a fatty acid- and retinoic acid-binding proptein. J Biol Chem 263: 14544–14551

Mayer JF Jr, Zirkin BR (1979) Spermatogenesis in the mouse. I. Autoradiographic studies of nuclear incorporation and loss of ^{3}H-amino acids. J Cell Biol 81: 403–410

Moriya M, Katagiri C, Yagi K (1993) Immuno-electron microscopic localization of calmodulin and calmodulin-binding proteins in the mouse germ cells during spermatogenesis and maturation. Cell Tissue Res 271: 441–451

Moriya M, Fujinaga K, Yazawa M, Katagiri C (1995) Immunohistochemical localization of the calcium/calmodulin-dependent protein phosphatase, calcineurin, in the mouse testis: its unique accumulation in spermatid nuclei. Cell Tissue Res 281: 273–281

Moss SB, Challoner PB, Groudine M (1989) Expression of a novel histone 2B during mouse spermiogenesis. Dev Biol 133: 83–92

Nakai K, Kanehisa M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14: 897–911

Narayanan V, Kaestner KH, Tennekoon GI (1991) Structure of the mouse myelin P2 protein gene. J Neurochem 57: 75–80

Nielsen SU, Pedersen AO, Vorum H, Brodersen R (1990) Fatty acid-binding protein from human heart localized in native and denaturing two-dimensional gels. Mol Cell Biochem 98: 119–125

Nielsen SU, Spener F (1993) Fatty acid-binding protein from rat heart is phosphorylated on Tyr^{19} in response to insulin stimulation. J Lipid Res 34: 1355–1366

Ogiwara A, Uchiyama I, Takagi T, Kanehisa M (1996) Construction and analysis of a profile library characterizing groups of structurally known proteins. Protein Sci 5: 1991–1999

Oko R, Clermont Y (1991) Origin and distribution of perforatorial proteins during spermatogenesis of the rat: an immunocytochemical study. Anat Rec 230: 489–501

Oko R, Morales CR (1994) A novel testicular protein, with sequence similarities to a family of lipid binding proteins, is a major component of the rat sperm perinuclear theca. Dev Biol 166: 235–245

Oko RJ (1995) Developmental expression and possible role of perinuclear theca proteins in mammalian spermatozoa. Reprod Fertil Dev 7: 777–797

Pearson RB, Kemp BE (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol 200: 62–81

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 2444–2448

Pouresmaeili F, Morales CR, Oko R (1997) Molecular cloning and structural analysis of the gene encoding PERF 15 protein present in the perinuclear theca of the rat spermatozoa. Biol Reprod 57: 655–659

Rajan N, Kidd GL, Talmage DA, Blaner WS, Suhara A, Goodman DS (1991) Cellular retinoic acid-binding protein messenger RNA: levels in rat tissues and localization in rat testis. J Lipid Res 32: 1195–1204

Ravindranath N, Papadopoulos V, Vornberger W, Zitzmann D, Dym M (1994) Ultrastructural distribution of calcium in the rat testis. Biol Reprod 51: 50–62

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmitt MC, Jamison RS, Orgebin-Crist MC, Ong DE (1994) A novel, testis-specific member of the cellular lipophilic transport protein superfamily, deduced from a complimentary deoxyribonucleic acid clone. Biol Reprod 51: 239–245

Seyedin SM, Kistler WS (1980) Isolation and characterization of rat testis H1t. An H1 histone variant associated with spermatogenesis. J Biol Chem 255: 5949–5954

Sutovsky P, Oko R, Hewitson L, Schatten G (1997) The removal of the sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. Dev Biol 188: 75–84

Trejo R, Delhumeau G (1997) Calmodulin content, Ca^{2+}-dependent calmodulin binding proteins, and testis growth: identification of Ca^{2+}-dependent calmodulin binding proteins in primary spermatocytes. Mol Reprod Dev 48: 127–136

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