Molecular Chaperone Calmegin Localization to the Endoplasmic Reticulum of Meiotic and Post-meiotic Germ Cells in the Mouse Testis*

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Summary. Calmegin is a testis-specific Ca\(^{2+}\)-binding protein that is homologous to calnexin. Recently, sperm from transgenic mice lacking calmegin have been shown to be infertile. To further characterize calmegin, we analyzed the precise stage of expression and the intracellular localization of this protein in germ cells during mouse spermatogenesis by an immunoperoxidase technique using the anti-calmegin monoclonal antibody TRA369. Light microscopic immunocytochemistry showed that calmegin appeared in early pachytenic spermatocytes, with the highest expression in round and elongating spermatids, and disappeared in the maturation phase of spermatids at step 15. Immunoelectron microscopy showed that selective localization was found at the endoplasmic reticulum membrane and the nuclear envelope of spermatogenic cells. During the maturation phase, a dramatic reduction in calmegin occurred in the endoplasmic reticulum of the spermatids, suggesting that the major function of calmegin has been completed by the time spermatids reach step 14. In addition, although the immunoreactivity was completely absent in the calmegin-deficient mutant mouse testis, ultrastructural analysis showed that mature sperm from the knockout mice were normal. This suggests that calmegin is not required for the morphogenesis of male germ cells. Thus, our results suggest that calmegin has a major role in mouse spermatogenesis, and also indicate that this protein would be useful as a maker molecule to study the functional role of the endoplasmic reticulum in the process of spermatid differentiation.

Mammalian spermatogenesis is a sequence of complex processes that involves the mitotic proliferation of spermatagonia, meiotic divisions of spermatocytes, and subsequent morphological changes in haploid spermatids into highly specialized sperm. In this process, many spermatogenic cell-specific proteins are synthesized under a controlled program of stage-specific gene expression (WILLISON and ASHWORTH, 1987; ERICKSON, 1990). During the post-meiotic germ cell development, termed spermiogenesis, spermatids undergo dramatic morphological transformations, and structural and biochemical modifications of the Golgi apparatus, centriolar-axonemal complex, mitochondria, and endoplasmic reticulum occur in the cytoplasm (FAWCETT and PHILLIPS, 1970; CLERMONT et al., 1993). In particular, the endoplasmic reticulum distributed throughout the cytoplasm aggregates and disappears during the late stages of spermiogenesis; this disappearance appears to have an important role in the process of spermatid differentiation (CLERMONT and RAMBOURG, 1978; NAKAMOTO and SAKAI, 1989).

Calmegin is a testis-specific Ca\(^{2+}\)-binding protein that has been characterized by a monoclonal antibody, TRA369, and is highly homologous to calnexin (WATA NABE et al., 1992, 1994). In contrast to other known chaperones, such as heat-shock proteins, BiP, and calreticulin, calmegin is a membrane-bound molecular chaperone of the endoplasmic reticulum (WADA et al., 1991; OU et al., 1993; JACKSON et al., 1994). More recently, it was shown that disruption of the calmegin gene resulted in failure to bind to the egg zona pellucida in sperm from calmegin-null mutant mice (IKAWA et al., 1997), suggesting that calmegin functions as a chaperone for sperm surface proteins that mediate the interactions between sperm...

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and egg, which are essential for sperm fertility in mice. In light of the calnexin function (BERGERON et al., 1994; ZHANG et al., 1997), it is possible that calmegin has a major role in quality control in the endoplasmic reticulum through the folding and assembly of functional proteins that are transported via the Golgi complex to the sperm surface during spermatogenesis. However, the substrate(s) for calmegin are not yet known. It is therefore important to identify the specific germ cells that express calmegin, because they also should be the cells expressing the substrate proteins.

In a previous immunofluorescence study using the TRA369 monoclonal antibody, calmegin showed stage-specific expression in germ cells of the mouse testes, specifically the pachyten spermocytes and spermatids (WATANABE et al., 1992). The purpose of the present study was to determine the precise stage of calmegin expression and its intracellular localization in the mouse testes using light and electron microscope immunocytochemical approaches. In addition, we examined the ultrastructure of testicular germ cells and epididymal sperm in calmegin-deficient mice.

MATERIALS AND METHODS

Animals
Male wild-type (+/+) 129/C57BL/6 mice and homozygous (−/−) calmegin-deficient littermates aged 12 to 16 weeks were used in this study. They were provided by Drs. Y. NISHIMUNE and M. OKABE (Osaka University, Japan). The generation of the calmegin knockout mice has been previously described (IKAWA et al., 1997). Investigations were conducted in accordance with the Guidelines for Animal Welfare of Miyazaki Medical College.

Monoclonal antibody
The anti-calmegin monoclonal antibody (TRA369, IgG1) used in this study was also provided by Dr. Y. NISHIMUNE. This antibody has been characterized by WATANABE et al. (1992).

Light microscopic immunocytochemistry
Mice were anesthetized with ether and the testes were fixed by intra-cardiac perfusion with 4% paraformaldehyde (PFA) or periodate-lysine-2% PFA (PLP) fixatives in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the testes were removed and further immersed in the same fixative for at least 4 h at 4°C. The tissues were washed in PB containing sucrose, and quickly frozen in OCT compound (Miles, Elkhart, IN, USA). Sections were cut at a 6-μm thickness on a Leitz cryostat (Model 1720), placed on silane-coated glass slides, and dried. Immunocytochemistry for light microscopy was carried out at room temperature as follows. After washing in phosphate buffered-saline (PBS, pH 7.4), the sections were treated with 0.3% hydrogen peroxide (H₂O₂) to block endogenous peroxidase activity. Non-specific binding was blocked with 2% normal goat serum, and the sections were then incubated with TRA369 at 1 : 50–1 : 100 dilutions from concentrated culture supernatants for 60 min. Control sections were carried out by replacing the first antibody with normal rat serum or PBS. Sections were washed in PBS and incubated with biotinylated rabbit anti-rat immunoglobulins (DAKO, Copenhagen, Denmark) diluted to 1 : 300 for 60 min, followed by horseradish peroxidase (HRP)-conjugated streptavidin (DAKO) to diluted 1 : 300 for 30 min. Immunoreaction was visualized with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ in 50 mM Tris buffer, pH 7.6. Stained sections were dehydrated through a graded ethanol series and xylene, and finally coverslipped.

Immuno electron microscopy
For immunocytochemistry at the electron microscopy level, PFA- or PLP-fixed cryostat sections were blocked with normal goat serum and incubated with TRA369 antibody at 4°C overnight. After washing, the sections were incubated with the HRP-conjugated Fab’ fragment of goat anti-rat IgG (Protos Immunoresearch, San Francisco, CA, USA) at a dilution of 1 : 50 for 2 h, fixed with 0.5% glutaraldehyde, reacted with a DAB-H₂O₂ solution, and post-fixed with 1% OsO₄. Control sections in which the primary antibody was omitted were processed in parallel. Sections were dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultramicro (Model 2088) and examined without counterstaining using a Hitachi H-7100 transmission electron microscope. The stages of the cycle of the mouse seminiferous epithelium were determined according to the morphological criteria by OAKBERG (1956) for light microscopy or DOOHER and BENNETT (1973) for electron microscopy.

Conventional light and electron microscopies
For conventional light microscopy, the testes were perfusion-fixed with Bouin’s fixative. The tissues were removed, further immersed overnight in the same fixative, dehydrated in a graded ethanol series, and embedded in paraffin. Five-micrometer paraffin sections were stained with periodic acid-Schiff (PAS)
reagent and hematoxylin. For conventional electron microscopy, the testes and epididymides were perfusion-fixed with 2.5% glutaraldehyde in PB. Mature sperm were collected from the cauda epididymidis into a mixture containing 2.5% glutaraldehyde and 2% tannic acid buffered with PB and fixed for 1 h. Washed samples were then post-fixed in 2% OsO₄, dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined using a transmission electron microscope.

RESULTS

Localization of calmodulin at the light microscopy level

In the wild-type mouse testis, immunoperoxidase staining with TRA369 antibody showed a distinct pattern dependent on the stage of the seminiferous cycle (Fig. 1). No immunoreaction was observed on spermatogonia or the early stages of primary spermatocytes (preleptotene, leptotene, and zygotene). A positive reaction was first faintly detected in early pachytene spermatocytes at stages I to III; it was observed over the cytoplasm but not in the nucleus

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Reactivity</th>
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<tbody>
<tr>
<td>Spermatogonia</td>
<td>–</td>
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<tr>
<td>Primary spermatocytes</td>
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<tr>
<td>Preleptotene</td>
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<td>Leptotene</td>
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<td>Zygote</td>
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<td>Pachytene</td>
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<td>Early</td>
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<tr>
<td>Middle</td>
<td>+</td>
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<tr>
<td>Late</td>
<td>+</td>
</tr>
<tr>
<td>Diplotene</td>
<td>+</td>
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<tr>
<td>Metaphase I</td>
<td>+</td>
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<tr>
<td>Secondary spermatocytes</td>
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<td>Metaphase II</td>
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<td>Spermatids</td>
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<tr>
<td>Steps 1-3 (Golgi phase)</td>
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<tr>
<td>Steps 4-7 (cap phase)</td>
<td>++</td>
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<tr>
<td>Steps 8-12 (acrosome phase)</td>
<td>++</td>
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<tr>
<td>Steps 13-14 (early maturation phase)</td>
<td>+, +/-</td>
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<tr>
<td>Steps 15-16 (late maturation phase)</td>
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- : negative, +/- : weak, + : medium, ++ : strong, +++ : very strong
At this stage, an intense reaction was observed in the cytoplasm of step 1-3 spermatids, whereas in the post-nuclear region of step 13-14 spermatids, a markedly lighter reaction was noted (Fig. 1a). Their antigenicity, however, appeared enhanced in immunoelectron microscopic sections post-fixed by the OsO₄ (see Fig. 6). During stages IV to VII, the cytoplasmic reaction became more pronounced in middle pachytene spermatocytes (Fig. 1b). At this stage, step 4-7 spermatids exhibited a very intense reaction in the cytoplasm, while no reaction was observed in step 15-16 spermatids (Fig. 1b). Released mature spermatids (sperm) as well as residual bodies were negative at stage VIII. During stages IX to XII, the intense reactivity remained in the post-nuclear region of steps 9-12 spermatids (Fig. 1c). At this stage, late pachytene, diplotene, and secondary spermatocytes exhibited the same intensity as middle pachytene spermatocytes (Fig. 1c). No immunoreactivity was observed in testicular somatic cells such as Sertoli cells, peritubular myoid cells, or interstitial Leydig cells. This results are summarized in Table 1. In brief, the TRA369 immunoreactivity appeared in early pachytene spermatocytes, with the highest expression in round and elongating spermatids, and disappeared in late maturation phase spermatids at step 15. Control sections did not show any significant reaction with any testicular cells.

In contrast to the wild-type mouse testes described above, no immunoreactivity could be observed in any sections from the calmegin-deficient mouse testes (Fig. 2a).

### Localization of calmegin at the electron microscopy level

To define the localization of calmegin in the spermatogenic cells, an immunoperoxidase electron mi-

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**Fig. 3.** Ultrastructural localization of calmegin in pachytene spermatocytes at stage IV of a wild-type mouse testis. Reaction products are evident at the membrane of the tubular endoplasmic reticulum and along the nuclear envelope (arrows), but not in the nucleus (N) or the plasma membrane, or in Sertoli cells (S). The open arrow indicates an intercellular bridge connecting meiotic spermatocytes, in which synaptonemal complexes (arrowheads) are visible. Bar=1 μm

**Fig. 4.** Ultrastructural localization of calmegin in a step 6 round spermatid of the wild-type mouse testis. Reaction products are seen on the endoplasmic reticulum encircling the surface of the Golgi apparatus (G) and the nuclear envelope (arrow), but not in the Golgi area, acrosomic granule (A), head cap (H), mitochondria (M), nucleus (N), or nuclear envelope (arrowheads) in contact with the acrosomic system. Bar=1 μm

**Fig. 5.** Ultrastructural localization of calmegin in a longitudinal section of step 11 elongating spermatids of a wild-type mouse testis. Reaction products are seen on long tubular endoplasmic reticulum along the inside and outside of the caudal tube (C), and around the axoneme (Ax), as well as on the tubular endoplasmic reticulum (arrow) under the cell surface. The nuclear envelope (arrowhead) of the spermatid is negative. N nucleus. Bar=1 μm
Figs. 3-5. Legends on the opposite page.
crosscopy technique was used. In the wild-type mouse testes, the antigen reacting with the TRA369 antibody was localized exclusively on the luminal side of the endoplasmic reticulum membrane of pachytene spermatocytes and spermatids. Consistent with our light microscopy findings, no significant reaction was observed in spermatogonia, the early stages of primary spermatocytes, or in testicular somatic cells.

In pachytene spermatocytes, immunoreaction products were localized to the tubular or spheroidal cisternae of the endoplasmic reticulum that were widely distributed throughout their cytoplasm, as well as to the nuclear envelope (Fig. 3). It was frequently observed that the calmodulin-positive endoplasmic reticulum of connected spermatocytes was continuous through the intercellular bridges (Fig. 3). No immunoreaction was observed in other cellular organelles such as the nucleus, mitochondria, Golgi apparatus, chromatoid body, or the plasma membrane.

During the Golgi and cap phases of step 1–7 spermatids, the patterns of the reaction were basically similar to those observed in the previous spermatocyte stages. However, the immunoreaction was not detected where the nuclear envelope contacted the acrosome, whereas it remained in the nuclear pore area that was not in contact with the acrosome (Fig. 4). At this stage, no reaction was found in the Golgi region or the acrosomic system, while the endoplasmic reticulum on the cis-side surface of the Golgi apparatus was reactive (Fig. 4).

During the acrosome phase, the cytoplasm of step 8–12 elongating spermatids contained abundant endoplasmic reticulum that showed regional changes or specialization. The reaction was localized to the endoplasmic reticulum which progressed toward the more distal portions of the cytoplasm, and not localized on the nuclear envelope (Fig. 5). In addition, the reaction could be seen at the long tubular endoplasmic reticulum cisternae, which were distributed around the manchette, just beneath the plasma mem-
brane, and along the cytoplasmic portion of the flagellum (Fig. 5). The three-dimensional arrangement of these endoplasmic reticulum structures has been described in detail by Clermont and Rambourg (1978).

During the early maturation phase, the reaction was localized to the endoplasmic reticulum, which was gradually condensed in a small area of the caudal cytoplasm of step 13–14 spermatids; most endoplasmic reticulum exhibited various irregular membranous profiles, some of which showed swollen, large, distended vesicles (Fig. 6). In contrast, no significant reaction was observed in the radial bodies or the annulate lamellae (Fig. 6).

During the late maturation phase, the reaction could no longer be seen in most of the regressed or degenerated endoplasmic reticulum of step 15 spermatid cytoplasm, but a very faint reaction remained in a few remnants of the endoplasmic reticulum (Fig. 7), which was undetectable at the light microscopy level. In step 16 spermatids, no reaction could be observed and, consistent with the finding of Nakamoto and Sakai (1989), we could not find endoplasmic reticulum in the cytoplasm.

In the testes of calmegin-deficient mice, no immunoreaction could be detected. Control sections did not show any significant reactivity except for an endogenous peroxidase activity in the matrix granules of some mitochondria.

Normal ultrastructure in the spermatogenic cells and epididymal sperm from calmegin-deficient mice

To determine whether a functional calmegin gene was required for the formation of normal spermatogenic cells or epididymal sperm, we compared the morphology of these cells in calmegin-deficient (+/−) mice with that in the wild-type (+/+ ) mice by conventional light and electron microscopy. Consistent with earlier observations (Ikawa et al., 1997), overall testicular organization and approximate numbers of germ cells within the seminiferous tubules were normal and indistinguishable between −/− and +/+ mice testes (Fig. 2b). Furthermore, ultrastructural analysis showed that no obvious mor-
phologic differences could be detected in spermatogenic cells or the epididymal sperm of −/− mice as compared with +/+ mice. Higher magnifications of mature sperm from −/− mouse epididymis showed a normal appearance, including the continuous plasma membrane covering the sperm head and tail, acrosomal membrane, and nuclear membrane (Fig. 8). The above conclusions on the morphological normality of sperm formation in −/− mice were based on observations of a total of 7 −/− and 3 +/+ mice.

**DISCUSSION**

The present study established that calmegin is localized at the endoplasmic reticulum membrane of meiotic and post-meiotic mouse spermatogenic cells and is especially abundant in the spermatids. Furthermore, the normal ultrastructure of calmegin-deficient mouse sperm was clearly shown. Thus, we confirmed earlier observations (Watanabe et al., 1992; Ikawa et al., 1997) and extended knowledge of calmegin expression and function at the light microscopy and electron microscopy levels.

Using the TRA369 monoclonal antibody, we showed that calmegin is expressed in pachytene stage spermatocytes, and that the expression persists until maturation phase spermatids. A similar expression pattern was recently reported by Ohsako and co-workers for calnexin-t (calmegin), which is recognized by IC9 monoclonal antibody, encoded by a gene identical to that for calmegen, and is expressed in spermatogenic cells of the hamster (Ohsako et al., 1994a) and mouse (Ohsako et al., 1998). However, there were some significant differences between TRA369 and IC9 antibodies: the IC9 reactivity was seen in middle pachytene spermatocytes until spermatids at step 16 (Ohsako et al., 1998), but the TRA369 reactivity was shown in early pachytene spermatocytes until spermatids at step 14. Although IC9 showed reactivity with both Bouin’s-fixed paraffin and PFA-fixed cryostat sections (Ohsako et al., 1994a, 1998), TRA369 did not exhibit any reactivities with the paraffin sections (data not shown) but did show reactivity with the cryostat sections. In addition, in the mouse testis, IC9 recognizes a 101-kDa protein (Ohsako et al., 1994a, b), while the molecular weight of the protein recognized by TRA369 is 93-kDa (Watanabe et al., 1994). Moreover, this 101-kDa protein has also been shown to change its molecular weight into 93-kDa when treated with acid phosphate (Ohsako et al., 1998), suggesting that IC9 may recognize the phosphorylated form of calmegin. From these lines of evidence, it is likely

**Fig. 8.** Conventional electron micrograph of a mature sperm from the cauda epididymis of a calmegin-deficient mouse. High power view of a frontal section through the sperm head, showing that overall components display normal morphologic features, including the plasma membrane (PM), the outer (OAM) and inner (IAM) acrosomal membranes covering the acrosome (A), and the nuclear membrane (NM) covering the nucleus (N). Bar = 0.1 μm
that conformations near the calmegin/calnexin-t-antigenic epitope influence the antigenic specificities of TRA369 and 1C9 differently.

Calmegin showed a distinct, developmentally controlled pattern of expression in the mouse testis, implying a regulatory role for calmegin during spermatogenesis. A weak expression was first apparent in the cytoplasm of early pachytene spermatocytes. Middle pachytene to diplotene spermatocytes exhibited a clear cytoplasmic expression that became more intense in post-meiotic spermatids up to step 12, suggesting a physiochemical change in the affinity of TRA369 for its epitope after meiotic division. During the maturation phase, calmegin was dramatically reduced in step 13-14 spermatids and completely absent from the final step spermatids (step 16), indicating a rapid and specific degradation of calmegin around step 15. Thus, these findings suggest that calmegin is synthesized during the formation of early pachytene spermatocytes to early maturation phase spermatids of the mouse.

Our immunoelectron microscopy demonstrated that the specific location of calmegin was the endoplasmic reticulum membrane and nuclear envelope, and that it was not found in other cellular organelles, including the Golgi apparatus, acrosome, and plasma membrane, providing the first definitive localization of calmegin in mouse spermatogenic cells. It is therefore possible to conclude that calmegin is an abundantly expressed protein that resides in the endoplasmic reticulum. Moreover, our data lend support to the conclusions of a previous molecular biological analysis (Watanabe et al., 1994) indicating that calmegin is a membrane-bound molecular chaperone in the endoplasmic reticulum. The nuclear envelope is known to undergo structural changes during spermiogenesis (Fawcett and Chemes, 1979; Clermont et al., 1993). We showed that calmegin was localized in the area of the nuclear pores of both pachytene spermatocytes and early stage spermatids, but not in the elongating spermatids or in the redundant nuclear envelope of later stage spermatids. This might be related to the observation that most transcription occurs during meiosis and in early spermatids (Hecht, 1993). In fact, the protein and the mRNA of the calmegin gene are detected simultaneously in pachytene spermatocytes and spermatids (Watanabe et al., 1994). Calmegin was also localized to the endoplasmic reticulum around the manchette during the elongating phase of spermatids. Although the manchette, which is made up of microtubules, is postulated to be closely associated with spermatid elongation (Fawcett et al., 1971; Russell et al., 1991), our present study showed a normal morphology for spermiogenesis in calmegin-deficient mice testes, suggesting the lack of any contribution of calmegin to the spermatid morphogenesis. However, we cannot totally exclude the possibility that other molecules may compensate for the putative role of calmegin in some interactions between the endoplasmic reticulum and manchette at the molecular level.

As the result of an extensive survey, we were able to detect a dramatic reduction of calmegin in the spermatid endoplasmic reticulum during the maturation phase. This coincides well with previous conventional electron microscopy finding on the regression and disappearance periods of the endoplasmic reticulum (Clermont and Rambourg, 1978; Nakamoto and Sawai, 1989). Importantly, we found that calmegin was localized to various forms of the endoplasmic reticulum at steps 13-14 but not to the degenerated endoplasmic reticulum at step 15. This suggests that a major function of calmegin has been completed by step 14 of mouse spermatids and that in the late maturation phase, calmegin, is undetectable and non-functional, even if the endoplasmic reticulum exists. Moreover, the present immunoperoxidase electron microscopy analysis demonstrated that no significant amount of calmegin was found in the radial body or the annulate lamella during the maturation phase. Thus, our results clearly demonstrate for the first time exactly when and where the functional regression of the endoplasmic reticulum occurs in the process of mouse spermiogenesis. We suggest that the radial body-annulate lamellae complex (Clermont and Rambourg, 1978) might be involved in the mechanism for the disappearance of the calmegin, depending on endoplasmic reticulum regression.

Finally, the ultrastructural studies presented here confirmed that there is no morphological difference in male germ cells between calmegin-deficient mice and wild-type normal mice. This is consistent with earlier light microscopy findings showing normal spermatogenesis in knockout mice, as well as normal viability and motility of their sperm (Ikawa et al., 1997). Taken together, the results suggest that calmegin is not required for either the formation of normal spermatogenic cells nor the maturation of epididymal sperm. In terms of its function as a chaperone, since calmegin-deficient mouse sperm failed to bind to the egg zona pellucida (Ikawa et al., 1997), calmegin substrate molecules in the endoplasmic reticulum are assumed to be transported to the sperm surface during spermiogenesis. Several proteins on the sperm surface have been reported as candidates for the zona pellucida adhesion molecules, including galactosyltransferase (GalTase) (Lopez et al., 1985; Gong et al., 1995), a 95-kDa protein (ZRK)
(LEYTON and SAILING, 1989; BURKS et al., 1995) and fertilin β (CHO et al., 1998). Interestingly, these proteins and/or mRNAs have been reported to be expressed in spermatocytes and early spermatids. In order to better understand the precise role of calmeigin during mouse spermatogenesis, it will be necessary to detect and characterize the substrate molecules for calmeigin.

In conclusion, the present immunocytochemical studies show that the endoplasmic reticulum of mouse pachytene spermatocytes and spermatids is the specific intracellular site of localization for calmeigin. Thus, calmeigin would be useful as a male germ cell-specific marker molecule to study the role of endoplasmic reticulum in the process of spermatid differentiation, and it should help in the diagnosis of spermatogenic disturbance.

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