Proc. Arthropod. Embryol. Soc. Jpn. 35, 7–11 (2000)

ISSN 1341–1527

Immunocytochemistry of cricket nebenkern with an antibody against PSTAIRE motif of CDKs

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Abstract

PSTAIRE motif-like immunoreactivity was detected in mitochondria of spermatocytes and spermatids of cricket, *Gryllus bimaculatus*, by immunocytochemical analysis with an antibody against conserved PSTAIRE motif found in Cdc2, CDK2 and CDK3. This immunoreactivity was located in the perinuclear region during the late meiotic prophase. In round spermatids, the immunoreactive substances became aggregated near the nucleus and formed a large spherical body. In later elongate spermatids, the immunoreactivities were observed as fine grains along the tails. Immunogold-electron microscopy of ultrathin sections of spermatids revealed that the distribution of gold labeling was restricted to a large mitochondrion, which is known as the nebenkern. Several immunoreactive bands were detected by immunoblot of the testis extract; the major band was estimated to be about 94 kDa.

Introduction

Spermatogenesis is an excellent example of cell differentiation. The male germ cells exhibit dramatic morphological changes, and various stage-specific proteins contribute to their development (Hecht, 1993). Our previous study on male germ cells of cricket, *Gryllus bimaculatus*, by means of lectin cytochemistry also showed the developmental changes of several kinds of glycoproteins (Suzuki and Nishimura, 1997a). To search for clues as to their function, and if possible, to select appropriate molecular markers that denote the cell state, we tested the immunoreactivities of cricket testis on several other antigens including cyclin-dependent kinases (CDKs). CDKs and their regulatory factors, cyclins, are key regulators of the cell cycle (Nigg, 1995). An immunocytochemical analysis by an antibody against the PSTAIRE motif, which is the conserved region of Cdc2 (CDK1) obtained in a broad range from yeast to human (Meyerson *et al.*, 1992), unexpectedly revealed the presence of the immunoreactivity in the "nebenkern" of cricket germ cells. The distribution pattern of the immunoreactive substance suggests its certain roles in the mitochondrial metamorphosis during insect spermatogenesis.

Materials and Methods

Crickets, *Gryllus bimaculatus*, were reared on an artificial mouse diet (Oriental Yeast Co. Ltd., Tokyo, Japan). Meiotic prophase is first observed in 5th instar and spermiogenesis can be found after 7th instar larvae (Suzuki and Nishimura, 1995, 1997b). Testes of 7th and 8th instar larvae were fixed with Bouin's fluid for light-microscopical immunocytochemistry. They were then dehydrated in ethanol and *n*-butanol, embedded in paraffin, and sectioned at 5 μm. For immunogold-electron microscopy, tissue processing by rapid-freezing and freeze-substitution was carried out according to Campbell *et al.* (1991) with a slight modification. Testis follicles of 7th instar larvae were incubated in insect saline (0.9% NaCl, 0.02% KCl, 0.02% $CaCl_2$) containing 15% 2,3-butanediol as cryoprotectant for 20–30 min on ice. They were then placed on a copper grid, the excess saline was removed, and plunged into liquid propane. Grids with cryofixed specimens were transferred into ethanol at −90℃ for freeze-substitution. After 5 days the specimens were passively allowed to warm to room temperature, removed from grids, and infiltrated with Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany). Polymerization was accomplished

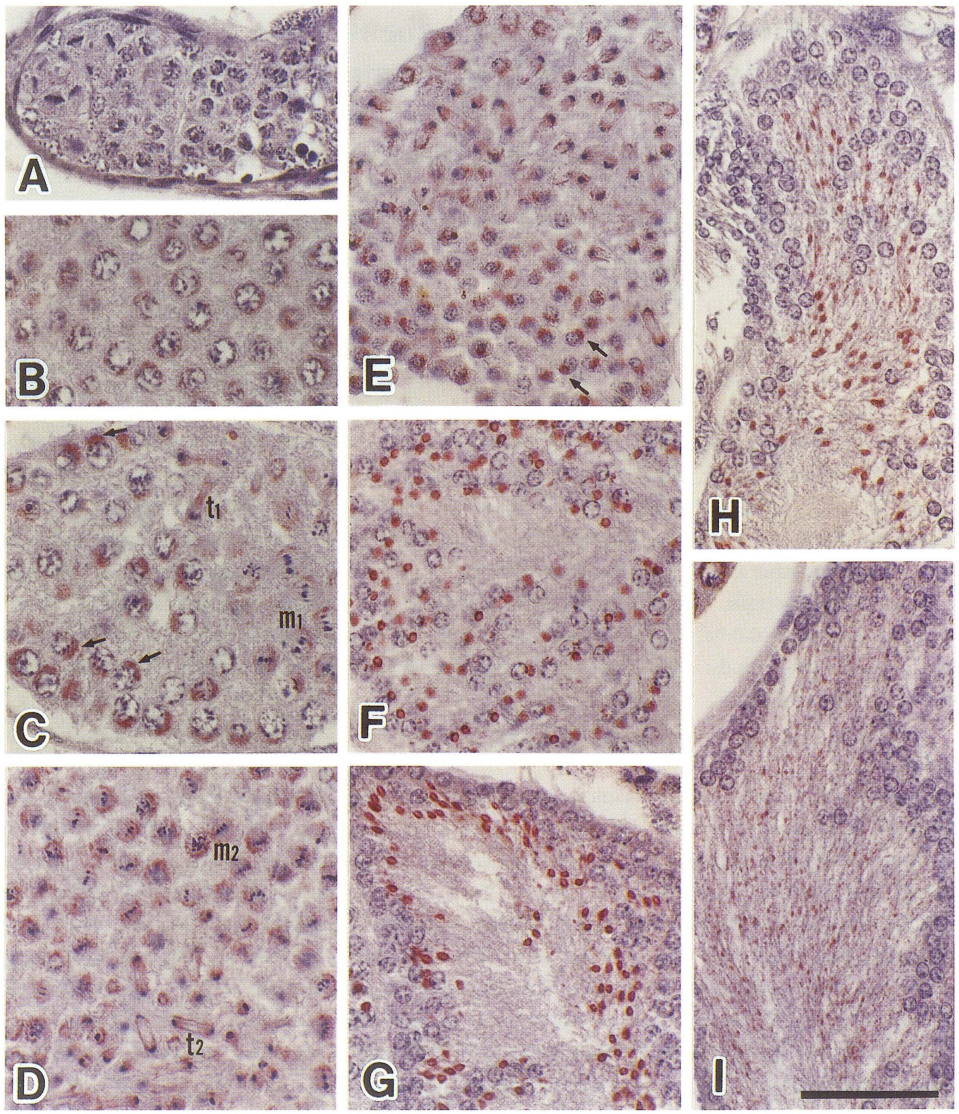

Fig. 1 Distribution of the PSTAIRE motif-like immunoreactivity during cricket spermatogenesis. The binding of anti-PSTAIRE antibody is detected as red color. Counterstained by hematoxylin. A. Spermatogonia. B. Mature primary spermatocytes. C. The first meiotic division. Primary spermatocytes at prometaphase, metaphase I (m_1) and telophase I (t_1) are co-located in a same cyst. Immunoreactive substances often show one-sided distribution in the prometaphase spermatocytes (arrows). D. The second meiotic division. m_2: metaphase II, t_2: telophase II. E. Meiosis II and round spermatids in a same cyst. Immunoreactive substances become aggregated in round spermatids (arrows). F. Young spermatids beginning tail elongation. A large spherical body behind each nucleus is immunostained. G, H. These bodies become elongate and located more posterior at later spermatids. I. Later elongate spermatid. The immunoreactivities are found as fine grains along the tails. Scale = 50 μm.

in BEEM capsules with UV irradiation for 24 h at −20℃.

An affinity purified rabbit anti-PSTAIRE antibody (Santa Cruz Biotechnology, Inc., California, USA) was used as the primary antibody for immunostaining procedure. This antibody was raised against 16 amino acid oligopeptide EGVPSTAIREISLLKE, which is conserved in Cdc2 kinases of yeast, *Drosophila*, mouse and human. This amino acid sequence is also conserved in other Cdc2-related kinases, CDK2 and

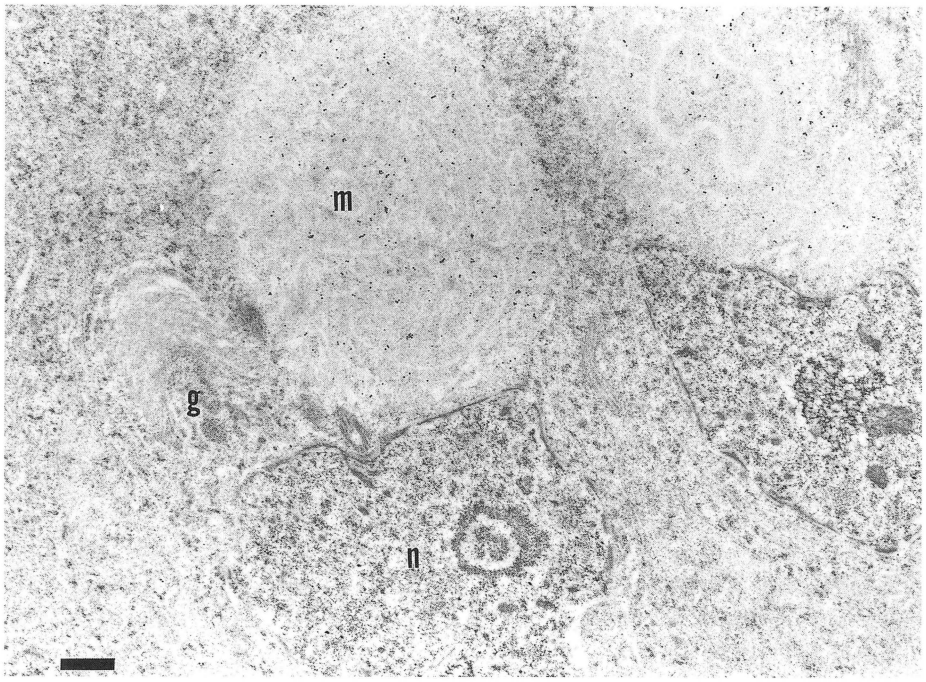

Fig. 2 Immunogold labeling of ultrathin sections of spermatids using the anti-PSTAIRE antibody. Gold particles are restricted in the nebenkern (m), which is formed by the aggregation of mitochondria. g: Golgi body, n: nucleus. Scale = 2 μm.

CDK3. As secondary antibodies, peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika Corp., West Chester, PA, USA) was used for light-microscopical immunocytochemistry, and goat anti-rabbit IgG antibody coupled to 20-nm colloidal gold (British Biocell, Cardiff, UK) was used for immunogold-electron microscopy. Incubation procedures were as described previously by Suzuki and Nishimura (1995). As a control, the primary antibody was preabsorbed with 10 μg/ml of the immunogen PSTAIRE peptide (Santa Cruz Biotechnology, Inc.) for 30 min before immunostaining.

Immunoreactive proteins were detected by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Testes of 7th instar larvae were dissected and homogenized with 10 volumes of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 12.5% glycerol, and 0.005% bromophenol blue) and the homogenate was boiled for 5 min. The sample was centrifuged and the supernatant (5 μl/lane) was subjected to SDS-PAGE (Laemmli, 1970) on 12% gel. The proteins were then electrotransferred onto PVDF membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) by semidry blotting procedure (Otto, 1993) and immunostained. Antibody reaction was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Biosource, California, USA) with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate.

Results and Discussion

Immunocytochemistry on a paraffin section of cricket testis using an anti-PSTAIRE antibody clearly showed its immunoreactivity in late spermatocytes and spermatids.

The staining was very faint, if any, in mitotic spermatogonia (Fig. 1A) and early spermatocytes. The immunoreactivity, located perinuclearly in the late meiotic prophase (Fig. 1B), became aligned on the lateral region of the spindle at metaphase I (Fig. 1C). In telophase I, the immunoreactive substances were distributed between the chromosomal aggregates (Fig. 1C). A similar distribution pattern of the immunoreactive substance was also observed during the second meiotic division (Fig. 1D). In round spermatids, the stained bodies aggregated near the nucleus. The co-localization of these spermatids and telophase II in a same cyst (Fig.

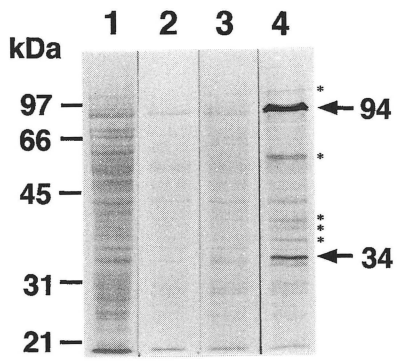

Fig. 3 Immunoblot analysis of the testis extract. Lane 1: total proteins stained by amido black 10B, lane 2: control staining without the primary antibody, lane 3: control staining with the primary antibody preabsorbed by the PSTAIRE peptide, lane 4: immunostaining with anti-PSTAIRE antibody.

1E) suggests the rapid coalescence of immunoreactive structures after meiosis II. The immunoreactive structures then formed a large spherical body beside the nucleus of each spermatid in which tail began to elongate (Fig. 1F). They became ellipsoidal and located more posterior (Fig. 1G, H), and in later elongate spermatids, the immunoreactivities scattered as fine grains along the tails (Fig. 1I). No response was detected in other parts of the testis. The immunostaining pattern was lost when the primary antibody was preabsorbed by the immunogen PSTAIRE peptide.

Distribution pattern of the immunoreactive substance observed in the paraffin section of cricket testis coincides with well known nebenkern behavior (von la Vatette St. George, 1886; Bowen, 1922; Fuller, 1993). Immunogold-electron microscopy of ultrathin sections using the anti-PSTAIRE antibody revealed that positive labeling was restricted to the nebenkern (Fig. 2). We recognize that this is the first report on the Cdc2-related immunoreactivity having these characteristics.

Several immunoreactive bands were detected by an immunoblot analysis of the testis extract from 7th instar larva (Fig. 3, arrows and asterisks). The 34 kDa band may be cricket Cdc2. The major band was, however, estimated to be about 94 kDa. The immunoresponse of this band was absorbed by the PSTAIRE peptide (Fig. 3, lane 3), suggesting that this protein, as well as the 34 kDa protein, may have the PSTAIRE motif-like structure, although it is yet to be elucidated whether the nebenkern immunoreactivity is due to the 94 kDa protein, and whether it belongs to so called CDK-family proteins.

The nebenkern is formed by the coalescence of all mitochondria in each spermatid. A predicted transmembrane GTPase, Fzo, is required for nebenkern formation in *Drosophila* (Hales and Fuller, 1997). In contrast to *Drosophila* Fzo, which appears just prior to fusion and disappears soon after its completion, the PSTAIRE motif-like immunoreactivity of cricket mitochondria is detected from the late meiotic prophase up to the elongate spermatid stage. During these stages, mitochondria align on the spindle, segregate to each spermatid, aggregate to form nebenkern, and elongate along the axoneme. Thus the distribution of the PSTAIRE motif-like immunoreactivity in cricket mitochondria throughout these stages may suggest its role accompanied with mitochondrial movement and metamorphosis during cricket spermatogenesis.

Acknowledgments: We are grateful to Dr. M. Obika, Keio University, for helpful discussions on electron microscopy. A part of this research was supported by a grant from Keio University.

References

Bowen, R.H. (1922) Studies on insect spermatogenesis. III. On the structure of the nebenkern in the insect spermatid and the origin of nebenkern patterns. *Biol. Bull.*, **42**, 53–85.

Campbell, S.S., B.J. Crawford and C.L. Reimer (1991) A simple ethanol-based freeze-substitution technique for marine invertebrate embryos which allows retention of antigenicity. *J. Microsc.*, **164**, 197–215.

Fuller, M.T. (1993) Spermatogenesis. *In* M. Bate and A. Martinez-Arias (eds.), *The Development of* Drosophila

melanogaster, *Vol. 1*, pp 71–147. Cold Spring Harbour Laboratory Press, New York.

Hales, K.G. and M.T. Fuller (1997) Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell*, **90**, 121–129.

Hecht, N.B. (1993) Gene expression during male germ cell development. *In* C. Desjardins and L.L. Ewing (eds.), *Cell and Molecular Biology of the Testis*, pp. 400–432. Oxford University Press, New York.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Meyerson, M., G.H. Enders, C.-L. Wu, L.-K. Su, C. Gorka, C. Nelson, E. Harlow and L.-H. Tsai (1992) A family of human cdc2-related protein kinases. *EMBO J.*, **11**, 2909–2917.

Nigg, E.A. (1995) Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bio Essays*, **17**, 471–480.

Otto, J.J. (1993) Immunoblotting. *Methods Cell Biol.*, **37**, 105–117.

Suzuki, A.C. and K. Nishimura (1995) Soybean agglutinin binding in germ cells during spermatogenesis of the cricket, *Gryllus bimaculatus*. *Zool. Sci.*, **12**, 591–597.

Suzuki, A.C. and K. Nishimura (1997a) Glycoconjugate profiles of insect spermatogenesis: lectin cytochemical analysis of the cricket, *Gryllus bimaculatus*. *Zool. Sci.*, **14**, 455–467.

Suzuki, A.C. and K. Nishimura (1997b) The testis development in 3rd- to 6th-instar nymphs of the cricket, *Gryllus bimaculatus*. *Zool. Sci.*, **14**, 637–639.

Von la Valette St. George (1886) Spermatologische Beiträge. Zweite Mittheilung. *Arch. Microsk. Anat.*, **27**, 1–12.