Outer Dense Fibers Serve as a Functional Target for Cdk5-p35 in the Developing Sperm Tail*

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Cdk5 is ubiquitously expressed in all tissues, but its activators, p35 and p39, are principally found in brain, and Cdk5 activity has mostly been associated with brain development, particularly neuronal differentiation and migration. Here we show that the p35 transcript and protein are also present in the testis, and an active Cdk5-p35 complex exists in this tissue as well. Cdk5 and p35 are prominently observed in elongating spermatid tails, particularly over the tail outer dense fibers (ODF). The appearance of Cdk5-p35 proceeds from the proximal to the distal end of elongating spermatids, coinciding with the proximal to distal assembly of ODF along the length of the tail axoneme. Incidentally, increased Cdk5-p35 activity is observed in isolated elongating spermatids and at a time when elongating spermatids appear in the developing testis, suggesting a role for Cdk5-p35 in spermiogenesis. The presence of Cdk5 and p35 in ODF isolated from rat spermatids implies a strong association among these proteins. In vitro ODF phosphorylation by Cdk5-p35 and decreased in vivo sperm tail ODF phosphorylation in p35-deficient mice indicate that Cdk5-p35 is an integral component of ODF and that ODF is a functional Cdk5-p35 target in the testis. Our results demonstrate for the first time that Cdk5-p35 may participate in the regulation of sperm tail development via a mechanism involving ODF phosphorylation. Apparently, as in brain development, Cdk5-p35 plays a part in testis development.

Activity of Cdk5-p35 is crucial for normal brain development. It has been demonstrated that brains of Cdk5−/− mice lack cortical and hippocampal lamination as well as cerebellar foliation (1). Mice deficient in p35 likewise show disruptions in cortical lamination but display only mild hippocampal defects and have a fairly normal cerebellum (2). There are indications that phenotypic alterations in Cdk5−/− or p35−/− mice are due to specific errors in neurite outgrowth, axon guidance, and neuronal migration (3, 4). Germ cell morphogenesis and migration during testis development are reminiscent of neuronal morphogenesis and migration during brain development. The spermiogenic phase of spermatogenesis, in which small round nondescript spermatids elongate and transform into highly specialized spermatozoa capable of coordinated swimming motions (5, 6), seems to particularly recapitulate the leading edge extension process and migration of neurons, which accounts for their proper positioning in the brain (7, 8).

In concurrence with its role in neuronal development, many of the identified Cdk5 substrates are cytoskeletal proteins such as neurofilament (9), actin-associated proteins such as Pak1 (10), and microtubule-associated proteins such as Map1b (11) and tau (12). Cdk5-p35 also phosphorylates Nudel, which interacts with the LIS1-dynein complex to regulate dynein-mediated axonal transport along microtubules (13). LIS1, the protein product of a gene mutated in lissencephaly, a human neuronal migration defect, can modulate microtubule organization and dynein distribution (14). Nudel, on the other hand, is a LIS1-interacting protein that has sequence homology to gene products implicated in fungal nucleokinesis (15). Both LIS1 and Nudel are enriched in neuronal growth cones (13). Thus, it appears that Cdk5-p35 contributes to the overall neuronal architecture and function through the regulation of cytoskeletal dynamics including those of actin, microtubules, and microtubule-associated proteins.

Spermiogenesis is characterized by major structural transformations in the round spermatid, including chromatin condensation, nuclear shaping, acrosome development, and tail formation. These transformations involve structures that are chiefly associated with spermatids such as the perinuclear theca, manchette, fibrous sheath, and outer dense fibers (ODF). The perinuclear theca is composed of a range of proteins of varying functions (16) and has been implicated in a number of essential cellular processes during spermiogenesis (17) and fertilization (16, 18). The manchette, comprised of a specialized microtubule array, has in particular been implicated in nuclear shaping (19). Although the sperm tail contains in its core a contractile axoneme composed of microtubules, similar to the motile cilia or flagella, it is far more complex than both. Indeed, the fibrous sheath, consisting of longitudinal columns and associated ribs, and the ODF, composed of nine coarse fibers, are the most prominent cytoskeletal elements of the tail and give the spermatozoa motile apparatus its distinctive morphological features (20). Thus, as in neuronal architecture and function cytoskeletal dynamics clearly play a major role in the morphogenesis and function of the spermatozoa.

Three ODF cDNAs from rat, encoding the 26-kDa ODF1 (51), 84-kDa ODF2 (21), and ~110 kDa ODF3 (22) proteins, have

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1 The abbreviations used are: ODF, outer dense fiber(s); RT, reverse transcription.
been cloned and characterized. However, analysis of the polypeptide composition of the rat ODF based on relative mobilities on polyacrylamide gels suggests the existence of six major and several minor polypeptides (23, 24). The major ODF polypeptides are rich in aspartic and glutamic acids, leucine, tyrosine, cysteine, glycine, serine, and proline (23, 25) and have abundant phosphoserine residues (23). Although previous studies indicate that ODF functions in maintaining the passive elastic structures and elastic recoil of the sperm tail (26), improving the bending torque of the tail (27) and/or protecting the sperm tail against shear forces during epididymal transport and ejaculation (28), the phosphorylation state of the ODF polypeptides may influence such functions and possibly affect sperm motility. Indeed, multiple protein kinases have been implicated in the activation of sperm tail motility (29, 30). However, to date, no specific kinase has been associated with sperm tail ODF phosphorylation. In this study we explored a potential function for the proline-directed serine/threonine kinase, Cdk5, in the developing testis, particularly in differentiating spermatids. We provide evidence that the Cdk5 activator, p35, and p35-associated Cdk5 activity exist in elongating spermatids and show for the first time that Cdk5/p35 is a sperm ODF kinase that may regulate sperm tail development.

**EXPERIMENTAL PROCEDURES**

**Animals and Antibodies**—Male Sprague-Dawley rats were obtained from the University of Calgary Biological Sciences Animal Facility. p35+/− and p39+/− breeding pairs that were kindly provided by Dr. Inez Vincent (University of Washington) and Dr. Li-Huei Tsai (Harvard University), respectively, were used to generate wild type, p35−/−, and p39−/− mice. The Cdk5 (polyclonal C-8 and monoclonal DC-17), p35 (C-19), cyclin D1 (72–13G), cyclin E (HE-12), and actin (I-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The proline-directed phosphothreonine antibody was from New England Biolabs Inc. (Beverly, MA). The ODF antibody was developed in the laboratory of Dr. Richard Oko (24).

**PCR/RT-PCR**—To detect the specific PCR products of Cdk5 (650 bp), p35 (268 bp), p39 (365 bp), and actin (414 bp), cDNAs generated from DNase 1-treated RNAs (isolated using Trizol, Invitrogen) by RT reaction were used as the template for PCR reactions. PCR was carried out using Taq from Invitrogen. The following primers were used: Cdk5 forward primer (TGAGGGTGTGCCAAGTTCAGC) and reverse primer (GGCATTGAGTTTGGGCACGAC); p35 forward primer (CAGGACCAAGGGTTTCATCACACC) and reverse primer (GTGGGTCAGCATTGACTGCAG), p39 forward primer (AGTCGCTTCGTGGGGACGAGCT), and reverse primer (AGTCGCTTCGTGGGGACGAGCT).
and reverse primer (GGTACGATCAGTCACGTGGGGC), β-actin forward primer (GAACCTTAACTCCTCCGCTT) and reverse primer (AGGAGAGATACCTGGAG). 

Northern Blotting—RNA was extracted using Trizol (Invitrogen). Blots were probed with radiolabeled RT-PCR product generated using the primers indicated above. DNA probes were radiolabeled by random priming (31) using the rediprime DNA labeling system (Strategene). Hybridization was carried out for 20 h at 65 °C in hybridization buffer containing 5× sodium chloride/sodium phosphate/EDTA or SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1–3× Denhardt’s solution, 0.1–0.5% SDS, 100–200 μg/ml denatured sheared salmon sperm DNA, and 5 M saturated tetrapyrrophosphate sodium salt in double distilled H2O. The blot was exposed to an x-ray film (Kodak Biomax-LS) for 24–72 h.

Immunohistochemistry, Immunogold Labeling, and Identification of Stages of the Cycle and Steps of Spermiogenesis—Immunohistochemistry, immunogold labeling, and identification of stages of the cycle and steps of spermiogenesis were performed as described previously (32).

Germ Cell Separation—Rat testes were decapsulated and incubated with collagenase (1 mg/ml) for 15 min at 37 °C, and the seminiferous tubules were incubated with trypsin (0.125 mg/ml) for 20 min at 37 °C. A single cell suspension was obtained by repeated pipetting of tubules and filtration through a 120-μm nylon mesh. Pachytyne spermatocytes, round spermatids, and elongating spermatids were then isolated by centrifugal elutriation (33, 34) of released germ cells.

Tissue Homogenization, Immunoprecipitation, SDS-PAGE, and Western Blotting—Tissue homogenates were prepared in 25 mM HEPES (pH 7.0, containing 250 mM NaCl, 1% Triton X-100, 1 mM diethiothreitol, 0.6 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml aprotinin, 0.3 mg/ml benzamidine, and 0.1 mg/ml soybean trypsin inhibitor) using a tissue homogenizer (Kinematica CH-6010 Kriens-LU, Switzerland). Samples were clarified by centrifugation at 16,000 × g for 40 min at 4 °C and filtration of supernatants through 0.45 μm filters. Tissue homogenates (1 mg) or gel filtration samples (500 μg) were precleared with protein A-agarose beads and incubated with fresh beads (preabsorbed with either anti-Cdk5 (C-8) or anti-p35 (C-19)) for 2 h on a rotating platform at 4 °C. The immunocomplexes were washed with Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.6 mM KCl) containing 0.1% Tween 20. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Millipore, Bedford, MA) and probed with the indicated primary antibody and corresponding horseradish peroxidase-conjugated secondary antibody (Pierce or Santa Cruz Biotechnology). Immunoreactive protein bands were visualized using the ECL detection system (Amer sham Biosciences).

Fig. 2. Cdk5 and p35 proteins are expressed in rat and mouse testes. Rat (A, lane 2) and mouse (B) testes homogenates (100 μg) were analyzed by Western blot analysis using Cdk5 (C-8), p35 (C-19), and actin (C-19) antibodies. Rat brain served as the positive control, and p35−/− mouse served as the negative control for p35. p25 is an active truncated form of p35. The asterisk denotes an intermediate processing product of p35. Wt, wild type, C, Western blot analysis of the p35 immunoprecipitates from rat testis (left panel) and p35 (right panel) protein expression in rat brain and testis (from the blot in Fig. 2A, upper and middle panels).

Fig. 3. p35-associated Cdk5 activity is present in testes. A, Cdk5 and p35 immunoprecipitates from rat testis homogenates (500 μg) were analyzed for histone H1 kinase activity. The remaining activity in blocked immunoprecipitates (ip) may be attributed to incomplete blocking by the peptide antigen. B, Western blot analysis of the p35 immunoprecipitate (lane 2) using a Cdk5 antibody (C-8); lane 1 is a blocked p35 immunoprecipitate. C-E, the specific activity of Cdk5-p35 in testis is comparable with that in brain. Cdk5-p35 was immunoprecipitated from rat testis (1) and brain (2) homogenates (500 μg) using a p35 antibody (C-19). C, histone H1 kinase activity of the immunoprecipitates (n = 3). D, corresponding Cdk5 immunoblot of one of the immunoprecipitates used in C, E, calculated Cdk5-p35-specific activity (pmol of PO42− incorporated into histone H1 peptide/min/Cdk5 level). Cdk5 levels (in square pixels) were determined by densitometric analysis using the NIH Image 1.61 program.
**Fig. 4. p35 and Cdk5 in the testis are distinctly localized to elongating spermatids.** Light micrographs of sections through rat seminiferous tubules immunoperoxidase-stained with p35 (A to F) and Cdk5 (G–J) antibodies. A, survey immunoperoxidase-stained testis section showing p35 immunoreactivity residing in elongated spermatids throughout the 14 stages of the cycle of the seminiferous epithelium (I–XIV). Note the intense immunostaining of spermatid tails in the tubular lumen as well as of Leydig cells (asterisk) in the interstitium. Sertoli cytoplasm immunostaining is also present in the pattern of streamers originating at the base and ending at the lumen of the tubules. B, higher magnification to show at this stage that the tail immunoreactivity is progressing distally in the lumen. C, spoke wheel immunostaining of Sertoli cytoplasm and intense staining of tails (arrowhead) within the lumen but no apparent staining of round spermatids (asterisk) between Sertoli cell streamers (arrows). D, at spermiation, the immunoreactive residual cytoplasm of the spermatids is phagocytosed as residual bodies (arrow) by Sertoli cytoplasm. The intense methylene blue counterstained clumps within the residual bodies are most likely accumulations of superfluous ribosomes. E, as shown in Fig. 1A, RT-PCR revealed the presence of transcripts for Cdk5 (arrow), but not p39 (lower left panel), in rat and mouse testes. These findings are indeed derived from p35 or p39 cDNAs from the RT reaction and not from potential genomic p35 or p39 DNA contamination. F, Cdk5 staining of Sertoli cell streamers (arrow). I, cross-section of sperm tails shows Cdk5 staining around the axoneme. J also shows the periaxonemal staining of Cdk5 (arrow) and staining of residual bodies (asterisk). Roman numerals in each tubule indicate the stage of the cycle of the seminiferous epithelium. Each scale bar = 10 μm. Note that immunostaining was abolished when the p35 or Cdk5 antibody was blocked with the peptide antigen used to generate the antibody (F’ and J’, respectively).

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**RESULTS**

**Cdk5 and p35, but Not p39, Are Expressed in Mouse and Rat Testes—**Initially we examined adult rat and mouse testes for Cdk5, p35, and p39 (a p35 isoform) transcript expression. As shown in Fig. 1A, RT-PCR revealed the presence of transcripts of Cdk5 (upper right panel) and p35 (upper left panel), but not p39 (lower left panel), in rat and mouse testes. These findings were supported by a parallel analysis of brain (positive control) and testes from p35−/− and p39−/− mice (negative controls for p35 and p39, respectively). Because p35 and p39 are intronless single exon genes, genomic DNA contamination will result in false positive PCR products. To prove that the PCR products are indeed derived from p35 or p39 cDNAs from the RT reaction and not from potential genomic p35 or p39 DNA contamination, isolated DNase 1-treated RNAs were used as template for each PCR negative control reaction (−), although the over-

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**Kinase Assays—**Histone H1 peptide (9–18) kinase activity of Cdk5 or p35 immunoprecipitates was determined as we described previously (35). The reaction was initiated by the addition of 100 μM [γ-32P]ATP and 100 μM histone H1 peptide (9–18) in the presence or absence of roscovitine (Calbiochem-Novabiochem). The amount of [γ-32P]phosphate incorporated into histone was measured using a Beckman LKB 1215 scintillation counter.

**Sperm Tail Isolation and ODF Purification—**Isolation of sperms and sperm tails and ODF purification were performed as described previously (32).

**Dephosphorylation and ODF Phosphorylation Assay—**Samples of purified ODF were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Dephosphorylation of immobilized ODF was performed as we described previously (32). Glutathione S-transferase-Cdk5 and glutathione S-transferase-p25 (2 μg each) were then added to kinase assay buffer (35), and phosphorylation of nitrocellulose-bound ODF was allowed to proceed for 1 h at RT in the presence of 100 μM [γ-32P]ATP. ODF phosphorylation by Cdk5/p25 was detected by autoradiography.
Spermatid purification is a 6–7-h procedure.

mRNA in rat brain is present at various developmental stages. By postnatal day, immunoblot analysis for Cdk5, p35, and cyclins D1 and E levels in testis at various developmental stages. C–D, round (lane/bar 1) and elongating (lane/bar 2) spermatids were isolated by centrifugal elutriation. C, immunoblot analysis of cell lysates (50 μg each fraction) for Cdk5 (upper panel) and p35/p25 (lower panel). p35 was converted to p25 during sample handling. Note that p35 is an unstable protein with a half-life of 20–30 min, and spermatid purification is a 6–7-h procedure. D, Cdk5-p35 immunoprecipitates (using the C-19 p35 antibody) from rat testis at various developmental stages.

In Cdk5/H18528 testes, analyses for histone H1 kinase activity (Fig. 3C) and p35 immunoprecipitates (using the C-10 p35 antibody) from each elutriation fraction (500 μg) were analyzed for histone H1 kinase activity.

Fig. 3—Significant Cdk5-p35 expression and activity in testis are associated with elongating spermatids. A, histone H1 kinase activity was assessed in Cdk5-p35 immunoprecipitates (using the C-19 p35 antibody) from rat testis at various developmental stages. P, postnatal day. B, immunoblot analysis for Cdk5, p35, and cyclins D1 and E levels in testis at various developmental stages. C–D, round (lane/bar 1) and elongating (lane/bar 2) spermatids were isolated by centrifugal elutriation. C, immunoblot analysis of cell lysates (50 μg each fraction) for Cdk5 (upper panel) and p35/p25 (lower panel). p35 was converted to p25 during sample handling. Note that p35 is an unstable protein with a half-life of 20–30 min, and spermatid purification is a 6–7-h procedure. D, Cdk5-p35 immunoprecipitates (using the C-10 p35 antibody) from rat testis at various developmental stages.
p35 and Cdk5 Activity in Testis Are Associated with Elongating Spermatids—Because the initial appearance of germ cells at a specific developmental stage corresponds to a specific age of development, we proceeded to investigate whether the observed p35 and Cdk5 staining in elongating spermatids coincides with the presence of p35-associated Cdk5 activity at a time when elongating spermatids start to appear in the developing rat testis. Fig. 5A shows relatively low Cdk5-p35 activity at 15–25 days postpartum, which corresponds to the age of appearance of primary zygotene and pachytene spermatocytes. Although Cdk5-p35 activity somewhat increased at P31 when round spermatids appeared, a significant increase in Cdk5-p35 activity was noted at P40 when elongating spermatids appeared and increased p35 level was observed (Fig. 5B). As expected, equivalent levels of the ubiquitously expressed Cdk5 was observed at different stages of development of the rat testis (Fig. 5B). No correlation between levels of cyclins D1 and E (Fig. 5B), which have been suggested to potentially activate Cdk5 in testis (40), and Cdk5 kinase activity (Fig. 5A) was noted. To further validate our assumption that Cdk5-p35 kinase activity in the developing testis is associated with elongating spermatids, round and elongating spermatids were isolated from P40 rat testes by centrifugal elutriation. As shown in Fig. 5C, Cdk5 and p25 (an active truncated form of p35) expression was higher in elutriated elongating spermatids (lane 2) compared with round spermatids (lane 1). Correspondingly, higher Cdk5-p35 activity was noted in the elongating spermatid fraction (Fig. 5D, bar 2) compared with the round spermatid fraction (Fig. 5D, bar 1). These findings correlate with our immunohistochemical data showing that Cdk5 and p35 are distinctly present in elongating spermatids. Together, these observations may suggest that p35-associated Cdk5 activity in testis has a role in spermiogenesis or differentiation of spermatids.

p35 and Cdk5 in Testis Are Specifically Associated with ODF—To determine the specific localization of p35 and Cdk5 in elongating spermatids, an ultrastructural analysis by electron microscopy was performed. Our results showed immunogold labeling of both p35 and Cdk5 over the ODF of steps 11–12 elongating spermatids (data not shown), with gold labeling becoming prominent in step 16 (Fig. 6, A and B). Consistent with immunoperoxidase staining, gold labeling became increasingly stronger as the spermatids mature, as seen in steps 17, 18, and 19 spermatids (Fig. 6, C–E, F, and G–I, respectively). In steps 18 and 19 spermatids, increasing p35 (Fig. 6F) and Cdk5 (Fig. 6H) labeling was also found in ODF regions closer to the peripheral microtubule doublets and dynein arms of the axoneme. In the cytoplasm, labeling was concentrated over granulated bodies (GB) as shown for Cdk5 in step 17 spermatids (Fig. 6E). In step 19 spermatids, labeling was observed in the remaining granulated bodies that are being discarded in the residual cytoplasm (Fig. 6G). In addition, labeling was seen over the connecting piece with clear gold particles over the basal plate, capitulum, and striated collar (Fig. 6I). These observations indicate that Cdk5-p35 is an ODF component.

ODF Is a Functional Cdk5-p35 Substrate in Testis—To further examine the link between ODF and Cdk5-p35, purified rat sperm tail ODF, which contained the major ODF polypeptides (Fig. 7A), were analyzed for Cdk5 and p35 association. As shown in Fig. 7B, both p35 (upper panel) and Cdk5 (lower panel) were found in sperm tail preparations (lanes 1 and 3) and purified ODF (lanes 2 and 4). We further sought to examine the significance of Cdk5-p35 and ODF association by analyzing whether Cdk5-p35 phosphorylates ODF. Because ODF isolation requires 1% SDS, which would inactivate ODF-bound Cdk5-p35, exogenous Cdk5-p35 was used in the assay. Autoradiography showed that the 20-, 26-, 32-, 56-, 80-, and 84-kDa ODF that were resolved by SDS-PAGE and transferred onto nitrocellulose were phosphorylated by Cdk5-p25 in the presence of [γ-32P]ATP/Mg2+ (Fig. 7C, lane 3). The significant difference in Cdk5-p25 phosphorylation of the non-dephosphorylated (lane 2) and dephosphorylated (lane 3) ODF samples supports the notion that a considerable amount of ODF peptides in the testis exist in a phosphorylated form.

Because Cdk5-p35, which we have now determined to be...
essentially associated with the major sperm tail structure ODF, is a proline-directed serine/threonine kinase, it would be interesting to compare the state of sperm tail proline-directed serine/threonine phosphorylation in wild type and p35-deficient mice. However, because commercially available phospho-serine and phosphothreonine antibodies do not produce convincing results, the significance of p35-associated Cdk5 activity in sperm tail ODF was examined by immunoblotting wild type and p35/H11002/H11002 mouse sperm tails with a reliable proline-directed phosphothreonine antibody. Fig. 7 shows a noticeable decrease in the level of proline-directed threonine phosphorylation in p35/H11002/H11002 mouse sperm tails (lane 2) compared with wild type (lane 1). Interestingly, the most prominent phosphorylated bands correspond in size to the 32- and 80-kDa ODF polypeptides (right panel), which form part of the main structure of sperm tails. It, thus, appears that Cdk5-p35 activity plays a part in the phosphorylation mechanisms in sperm tails.

DISCUSSION

Cdk5 activity has mostly been associated with brain, but among other tissues examined the testis has the next most abundant level of Cdk5 (38, 39). However, the rapid influx of exciting findings on the role of Cdk5-p35 in brain development has overshadowed the progress on Cdk5 studies in testis. This was sustained by the lack of evidence for the presence of a Cdk5 activator in the testis. Cdk5 activity has been reported in Leydig (TM3) and Sertoli (TM4) cell lines (40), but kinase activity has not been linked to p35 or its isoform, p39. Instead, Cdk5 activity has been correlated with levels of cyclins D1 and E, but interaction between Cdk5 and cyclin D1 or E or cyclin D1/E-associated Cdk5 activity has not been demonstrated. In this study, we provide evidence that the Cdk5 activator, p35, as well as p35-associated Cdk5 activity exist in the testis. Conversely, no correlation was detected between the level of cyclin D1 or E and increasing Cdk5 activity during testis develop-
ment. Indeed, it was previously reported that although Cdk5 could interact with cyclins D and E, the resulting complexes do not possess catalytic activity, and their specific function remains ambiguous (41, 42). In the testis, although the Cdk5 and p35 protein levels are less abundant compared with those in brain, the specific activity of Cdk5-p35 is comparable in these tissues. Thus, it appears that Cdk5-p35 activity has a relevant role in the testis.

Immunolocalization of p35 and Cdk5 in elongating spermatids with apparently greater labeling intensity of both proteins in later steps of development suggest a role for Cdk5-p35 in spermatid differentiation or spermiogenesis. Increased p35 level and Cdk5-p35 activity in rat P40, when elongating spermatids begin to appear in the developing testis, and increased Cdk5-p35 expression and activity in elutriated elongating spermatids support this premise. Furthermore, immunolabeling of p35 clearly proceeds from the proximal to the distal end of elongating spermatids following the proximal to distal assembly of the cytoskeletal ODF along the length of the microtubule-elongating spermatids (32). A relationship between Cdk5-p35 and ODF formation and/or function is also supported by Cdk5-p35 localization to granulated bodies, which presumably serve as storage sites for proteins destined to form ODF (43). Indeed, the presence of Cdk5-p35 in ODF purified via a stringent procedure that includes 1% SDS treatment implies a tight association between the kinase and ODF and may suggest a functional relationship between these proteins. This theory concurs with the fact that the six major fiber components of ODF in rats are highly phosphorylated at serine residues (23). In addition, several serine/threonine-proline residues are present in ODF. It should be noted, however, that phosphorylation by Cdk5-p35 at a serine/threonine residue in vivo can occur in the absence of proline and/or a basic residue at the +2 position. For example, although it appears that tau is a poor substrate for Cdk5 since many of the tau phosphorylation sites are not the primary substrate determinant for the kinase (Ser/Thr-Pro-X-Lys/Arg/His (44)), it was found that sulfated glycosaminoglycans such as heparin and heparan sulfate dramatically enhance tau phosphorylation by Cdk5 (12 versa 3.8 mol of phosphate/mol of tau (45)). Heparin facilitates tau phosphorylation by Cdk5 through alteration of the primary substrate determinant from Ser/Thr-Pro-X-Lys/Arg/His to Ser/Thr-Pro (44). In the presence of heparin, tau is also phosphorylated by Cdk5-p35 at Ser-262, a non-proline-directed site (45).

Because ODF has been implicated in maintaining the sperm tail structure (26–28), it is possible that ODF phosphorylation by Cdk5-p35 regulates such function and, consequently, influences sperm motility. However, based on the Cdk5-p35 immunolabeling data discussed above, it is likely that Cdk5-p35 interaction with and phosphorylation of the ODF polypeptide components function to regulate the transport and/or assembly of ODF and/or its association with the axoneme during spermatid tail development. In the later steps of spermatid elongation, Cdk5 and p35 were also noted in closer proximity to the area of the axoneme peripheral microtubule doublets and dynein arms. Potentially, some of the ODF-associated Cdk5-p35 serve as reservoirs for the kinase that probably regulates the microtubule and dynein-mediated movement/bending of sperm tails. This assumption also emerged from our separate investigations showing that dynein associates with Cdk5-p35 in rat sperms (data not shown). Although we cannot completely rule out the possibility of an indirect effect by Cdk5-p35 on ODF phosphorylation in vivo, our in vitro data indicate that Cdk5-p35 can cause direct phosphorylation of ODF. In addition, our hypotheses are in accord with the regulation of cytoskeletal dynamics by Cdk5-p35 (through phosphorylation of its cytoskeletal and cytoskeleton-associated substrates) during neuronal differentiation (46) and migration (47). Incidently, neuronal and spermatid differentiation both involve morphological changes that include extension/elongation. Furthermore, migration/motility by differentiated neurons and sperms is required to accomplish their function in brain and testis development, respectively. Evidently, as in brain development, Cdk5-p35 plays a part in testis development. In the testis, Cdk5-p35 seems to participate in the regulation of sperm tail development via ODF phosphorylation. This concurs with recent findings that Cdk5-p35 plays a significant role in the differentiation of non-neuronal cell types such as muscle cells (48), lens fibers (49), and promyelocytic cells (50).

Our discovery that the p35-associated Cdk5 activity exists in the developing testis and the identification of ODF as a likely target of Cdk5-p35 in vivo serve as relevant novel findings that will be useful for further characterization of the role of Cdk5-p35 and phosphorylated ODF in the testis. Indeed, although ODF is known to be highly phosphorylated in vivo, this study provides both in vitro and in vivo evidence for Cdk5-p35 as the first kinase identified thus far that causes sperm tail ODF phosphorylation. Indication that Cdk5-p35 is an integral component of ODF and determination of a functional interaction between these proteins could also provide further insight into signaling pathways that lead to sperm tail development and, possibly, motility.

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Outer Dense Fibers Serve as a Functional Target for Cdk5-p35 in the Developing Sperm Tail

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