Microtubule-independent and Protein Kinase A-mediated Function of Kinesin KIF17b Controls the Intracellular Transport of Activator of CREM in Testis (ACT)*

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Kinesins are motor proteins that transport their cargos along microtubules in an ATP-dependent manner. The testis-specific kinesin KIF17b was shown to directly regulate cAMP-response element modulator (CREM)-dependent transcription by determining the subcellular localization of the activator of CREM in testis (ACT), the testis-specific coactivator of CREM in postmeiotic male germ cells. CREM is a crucial transcriptional regulator of many important genes required for spermatid maturation, as demonstrated by the complete block of sperm development at the first steps of spermiogenesis in crem-null mice. To better understand the complex regulation of postmeiotic germ cell differentiation, we further characterized the ACT-KIF17b interaction, the function of KIF17b, and the signaling pathways governing its action. In this study, we demonstrated that the abilities of KIF17b to shuttle between the nuclear and the cytoplasmic compartments and to transport ACT are neither dependent on its motor domain nor on microtubules, thus revealing a novel microtubule-independent function for kinesins. We also showed that the cyclic AMP-dependent protein kinase A mediates the phosphorylation of KIF17b, and this modification is important for its subcellular localization. These results indicate that cyclic AMP signaling controls CREM-mediated transcription in male germ cells through modification of KIF17b function.

The complex differentiation of spermatogenic stem cells into mature sperm is governed by a highly specialized and strictly controlled program of gene expression. In spermatogonia and spermatocytes, the genes required for accurate mitotic and meiotic functions, respectively, are transcribed, Transcription ceases during the homologous chromosome pairing and meiotic recombination, but is again activated in postmeiotic germ cells. In the course of the differentiation of haploid spermatids into mature sperm, the genes required for development of acrosome, tail formation, removal of cytoplasm, and compaction of DNA are expressed (1–3). Upon compaction of chromatin by replacement of histones with transition proteins and protamines, the transcriptional activity ceases, and the mature sperm is transcriptionally inactive (4, 5). The transcription factor cAMP-response element modulator (CREM) protein is highly expressed in male germ cells (6, 7) and regulates the expression of many important postmeiotic genes, such as genes encoding protamines and transition proteins (8). Disruption of the crem gene in the mouse blocks the development of germ cells at the first step of spermiogenesis (9, 10), indicating the crucial role of CREM in postmeiotic germ cell differentiation.

CREM-dependent transcription in testis is regulated by the testis-specific LIM-only protein activator of CREM in testis (ACT) that functions as a coactivator for CREM (11). The phenotype of act-null mice indicates that ACT is involved in the control of a specific subset of CREM target genes (12). The study of act-null mice demonstrated that ACT is required for the normal development of sperm head and tail. ACT is expressed at specific stages of germ cell development, the highest level of expression occurring in postmeiotic haploid round spermatids (11). The subcellular localization of ACT is regulated by a kinesin motor protein, KIF17b, which colocalizes with ACT in haploid spermatids and mediates the transport of ACT from the nucleus to the cytoplasm at specific stages of spermatid maturation (13). The expression of ACT in testis remarkably overlaps with CREM expression, and relocation of ACT to the cytoplasm by the action of KIF17b correlates with the termination of transcription of the CREM-regulated genes (11, 13).

Spermatogenesis is controlled by a complex network of endocrine, paracrine, and autocrine signals. In response to the hypothalamic gonadotropin releasing hormone, the pituitary gland secretes two hormones, luteinizing hormone, and follicle-stimulating hormone (FSH), that are involved in the regulation of spermatogenesis. Luteinizing hormone regulates the testosterone secretion by somatic Leydig cells located in the interstitial tissue of the seminiferous tubules. FSH acts on Sertoli cells, the only somatic cells inside the seminiferous tubule, by stimulating signaling, gene expression, and secretion of peptides and other signaling molecules (14). Germ cells lack the receptors for pituitary hormones and are embedded in cytoplasmic pockets of Sertoli cells, which control spermatogenesis through strict paracrine regulation. To respond to external stimuli, germ cells utilize various transduction pathways that together form a complex signaling network governing germ cell development. The signaling mechanisms transferring information

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The abbreviations used are: CREM, cAMP-response element modulator; ACT, activator of CREM in testis; cAMP, cyclic AMP, FSH, follicle-stimulating hormone; PKA, protein kinase A; AC, adenylate cyclase; DAPI, 4′,6-diamidino-2-phenylindole.
PKA-mediated Control of Kinesin KIF17b in Testis

between Sertoli cells and germ cells remain so far largely unknown (15).

KIF17b is shutting between the nuclear and cytoplasmic compartments and is able to transport ACT from the nucleus to the cytoplasm at a specific stage of spermatid differentiation (stage VIII/IX). The signals in haploid germ cells that induce KIF17b to relocate with ACT to the cytoplasm after the onset of spermatid elongation are yet unknown. In this study, we have clarified the function of KIF17b and the regulation of CREM-dependent transcription by studying the mechanisms that modulate KIF17b localization. Strikingly, movement of KIF17b between nucleus and cytoplasm and its ability to transport ACT were shown to be independent of microtubules and the motor domain of the kinesin. These results indicate a novel, microtubule-independent mechanism for the function of kinesins. We also demonstrated that KIF17b movement is modulated by the cyclic AMP-dependent protein kinase A (PKA) dependent on phosphorylation, suggesting a role of cyclic AMP signaling in the regulation of CREM-dependent transcription in male germ cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pGAD-ACT, pSG5-ACT, and pCS2-MTK-ACT, containing the N-terminal Myc tag, have been described previously (11). pSG5-KIF17b has been described previously (13). KIF17b fragments were generated by PCR using the primers containing specific restriction sites. They were cloned either in pGHT9 yeast expression vector (Clontech), containing the Gal4 DNA-binding domain (EcoRI and SalI), or in pT1 vector, which is the pSG5 vector with a modified multiple cloning site (EcoRI and HindIII). KIF17b fragments were also transferred from pT1 vector to pCS2-MTK vector (16), containing the N-terminal Myc tag, using EcoRI and XhoI restriction sites. The full-length KIF17b was generated by digesting the N-terminus of KIF17b from the pSG5 vector by EcoRI and BstEII (there is an internal BstEII site in the KIF17b sequence) and ligating it to the KS2-MTK-KIF17b-C terminus digested with the same enzyme. The serine residues within the putative PKA consensus sites were mutated to alanines using the site-directed mutagenesis kit (Stratagene) and pCS2-MTK-KIF17b as a template.

Yeast Interaction Assays—Yeast transformation and the β-galactosidase assays were performed in yeast strain Y190, as described in the Clontech Matchmaker two-hybrid system protocol. Yeast cells were co-transformed with the pGBT-KIF17b constructs pGAD-ACT and β-galactosidase reporter. β-Galactosidase activities were calculated in Miller units. The results reported are the means of three independent experiments.

Immunofluorescence and Treatments—COS-1 cells grown on 8-cm culture dishes were transfected by FuGENE transfection reagent (Roche Applied Science) with the indicated plasmid, the total amount of DNA being 1 µg. 32 h after transfection, the cells were treated for either 6 h with 25 µM colchicine (Sigma) or 1 h with signalizing pathway inhibitors (10 µM H-89, 20 µM PD98059, 20 µM SB203580, 5 µM Ro-31–8220, 200 µM SQ22536, or 10 ng/ml TNFα B (Calbiochem)). After the treatments, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min, and nonspecific sites were blocked with 5% bovine serum albumin for 1 h. The immunofluorescence analysis was performed using polyclonal K3638 anti-KIF17 antibody, monoclonal anti-ACT antibody (1:1000), or monoclonal anti-β-tubulin antibody (1:1000) (Chemicon International, Temecula, CA). AlexaFluor goat anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. AlexaFluor goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Immunocomplexes were detected by enhanced chemiluminescence (Pierce).

In Vivo Phosphorylation—Overexpressed Myc-tagged KIF17b was immunoprecipitated from COS-1 cells using the anti-Myc 9E10 antibody and protein G-Sepharose (Amersham Biosciences). In vitro phosphorylation was performed in a buffer containing 50 mM HEPES, 100 mM KCl, 0.5% Nonidet P-40, 5 mM NaF, 1 mM dithiothreitol, 50 µM cold ATP, 5 µCi of [γ-32P]ATP (Amersham Biosciences), and 0.05 units/µl catalytic subunit of PKA (Sigma) in a volume of 20 µl at 37 °C for 20 min. In some cases, mouse testis protein extract was used instead of PKA. The reaction was stopped by adding 20 µl of Laemmli sample buffer. The samples were run into a SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue dye to confirm equal loading of PKA and substrates. The gel was dried and autoradiography was performed to detect phosphorylation. A small part of the gel was cut out and run into an SDS-polyacrylamide gel and immunoblotted with anti-Myc 9E10 antibody to confirm equal amounts of KIF17b in each lane.

Preparation of Testis Extracts—To prepare testis extracts, the testes were decapsulated, and the seminiferous tubules were homogenized in a buffer containing 170 mM NaCl, 50 mM Tris-HCl, pH 8, 0.5% Nonidet P-40, 5 mM EDTA, 20 mM NaF, 1 mM dithiothreitol and 1:1000 protease inhibitor mixture. The lysate was centrifuged for 30,000 revolutions/min for 15 min. The protein concentration was measured with the Bio-Rad protein assay reagent and adjusted to 5 µg/µl.

RESULTS

The Integrity of LIM Domains of ACT Is Required for the Interaction with KIF17b—KIF17b was originally found as an interaction partner of ACT in a yeast two-hybrid screening using full-length ACT as a bait (13). We have performed deletion analyses and yeast two-hybrid interaction assays to characterize the regions of ACT and KIF17b involved in the interaction. ACT is a LIM-only protein containing four and a half LIM domains, each consisting of the two sequential zinc fingers (11, 17). Deletion analysis of ACT demonstrated that none of the LIM domains alone mediates the interaction with KIF17b, but it is rather the integrity in the organization of the domains that is required for the association (Fig. 1A). Deletion of the half-LIM domain at the N terminus of ACT or the first full LIM domain reduced the interaction to 40 or 70%, respectively. All other deletions disrupted the interaction almost completely. These results indicated that the general three-dimensional ACT structure, rather than a specific domain, was responsible for the interaction with KIF17b.

The Central Region of KIF17b Interacts with ACT—KIF17b has an N-terminal motor domain that is very well conserved between all kinesin family members (18). Located in the C terminus is a highly variable domain that confers a cargo-binding function to many kinesins (19). In the central region of KIF17b, there is a long stalk region containing two coiled-coil domains. This region is known to be a regulatory region controlling, for example, kinesin dimerization (18). Interestingly, the original KIF17b clone found in the two-hybrid screen spanned this region, covering amino acids 320–720 of the KIF17b sequence (13). The role of this region in the interaction with ACT was confirmed in the yeast interaction assays using full-length ACT and KIF17b deletion mutants. In this assay, the C-terminal region (residues 720–1038 of KIF17b) that corresponds to the classical cargo binding region does not bind to ACT (Fig. 1B). In contrast, the interaction is mediated by the central stalk region of KIF17b (Fig. 1B). Detailed deletion analysis demonstrated that residues 520–620 are essential for the binding of KIF17b to ACT (Fig. 1, B and C).
The Ability of KIF17b to Transport ACT Is Independent of the Motor Function—Because the traditional function of kinesins is to carry cargos along microtubules in an ATP-dependent manner, we wanted to investigate whether microtubules and the motor function of KIF17b are needed for its ability to shuttle between the nucleus and the cytoplasm and thereby to transport ACT across the nuclear membrane. First, we studied the subcellular localization of full-length KIF17b and of a deletion lacking the N-terminal motor domain (KIF17b-(320–1038)). KIF17b constructs were cotransfected with ACT into COS-1 cells, and immunofluorescence was performed using specific antibodies. As reported previously, KIF17b is able to shuttle between the nuclear and cytoplasmic compartments and to transport ACT from the nucleus to the cytoplasm (13). Wild type KIF17b is mainly cytoplasmic, but a small percentage of the protein is either predominantly in the nucleus or equally distributed in the nucleus and cytoplasm (Fig. 2A). When coexpressed in the same cell, ACT follows the localization pattern of KIF17b, as confirmed by labeling cells with the
antibody raised against ACT (13) (data not shown). As KIF17b-(320–1038) is found both in nuclear and cytoplasmic compartments, it is evident that deletion of the motor domain does not change the ability of KIF17b to shuttle (Fig. 2, B and C). Nuclei were stained by DAPI to highlight the different localization patterns of KIF17b. A mutant of KIF17b lacking the motor domain still colocalizes with ACT and transports ACT through the nuclear envelope (Fig. 2, B and C). These results indicate that the motor function is not required for KIF17b shuttling or for its ability to determine subcellular localization of ACT. The microtubule independency of KIF17b shuttling was further confirmed by treating cells with a microtubule-depolymerizing drug, colchicine, before immunofluorescence. After the treatment, all microtubules were disrupted, as shown by immunostaining with anti-tubulin antibody. However, KIF17b was still able to shuttle (Fig. 2, D and E) and colocalize with ACT in both nucleus and cytoplasm (Fig. 2F).

**PKA Regulates the Subcellular Localization of KIF17b**—To identify the signaling pathways involved in regulating KIF17b function, cultured cells overexpressing KIF17b and ACT were treated with various inhibitors to block specific signaling pathways. The percentages of cells expressing KIF17b in the cytoplasm or in the nucleus or diffusely in both compartments were calculated. Normally, 70% of cells express KIF17b predominantly in the cytoplasm. The rest of the cells express KIF17b either mainly in the nucleus or diffusely in both compartments (Fig. 3A, column 1). The protein kinase C inhibitor (Ro-31–8220), mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor (PD 98059), p38 kinase inhibitor (SB 203580), or the inhibitor of small Rho GTPases (Toxin B) had no effect on the localization of KIF17b (Fig. 3A). In contrast, the protein kinase A inhibitor H-89 drastically changed KIF17b localization, decreasing the cytoplasmic fraction of KIF17b and increasing the number of cells expressing KIF17b diffusely in both nuclear and cytoplasmic compartments (Fig. 3A). The ability of KIF17b to interact and colocalize with ACT was not affected by H-89, as shown by coimmunoprecipitation and immunofluorescence experiments (data not shown and Fig. 3B). These results strongly suggest that PKA is regulating either the cytoplasmic retention of KIF17b or the transport of KIF17b through the nuclear envelope. The adenylate cyclase inhibitor SQ22536 had a similar effect, further confirming the involvement of cyclic AMP and PKA in the regulation of KIF17b movements (Fig. 3C).

**KIF17b Is Phosphorylated in Cells**—Next we wanted to know whether the effect of H-89 on KIF17b localization is mediated by its phosphorylation. Many kinesin family members have been reported to be phosphorylated by various kinases, but whether KIF17b is a phosphoprotein remained unknown. In vivo phosphorylation experiments were performed to investigate whether KIF17b becomes phosphorylated in cultured cells. 36 h after transfection, cells were incubated in a phosphate-free medium with $^{32}$P orthophosphate. After immunoprecipitation, phosphorylation of KIF17b was readily detected by autoradiography (Fig. 4A). KIF17b seems to be heavily phosphorylated; both the C terminus and the central region of KIF17b are independently phosphorylated in cells, suggesting the presence of several independent phosphorylation sites (Fig. 4, B and C). KIF17b lacking the N-terminal motor domain seems to be a better substrate for phosphorylation than the full-length protein, indicating a possible role for the motor domain in the control of kinesin phosphorylation.

**PKA Is Required for KIF17b Phosphorylation in Cells**—To study the involvement of various signaling pathways in the phosphorylation of KIF17b, cultured cells overexpressing KIF17b were treated with signaling inhibitors during the incubation with $^{32}$P orthophosphate. Interestingly, treatment of the cells with H-89 (but not with the other inhibitors) blocked the phosphorylation of KIF17b (Fig. 5A). This correlates with the effect of H-89 on KIF17b localization and strongly suggests that PKA mediates the phosphorylation of KIF17b in cells. KIF17b is predominantly expressed in testis (13). To investigate whether KIF17b can also be phosphorylated in testis, we performed the kinase assay using KIF17b immunoprecipitated from cultured cells as a substrate and testis extract as a source of kinases. KIF17b was shown to be phosphorylated after incubation with testis extract in the presence of $[^{32}$P]ATP, thus demonstrating that there are active kinases capable of phosphorylating KIF17b in male germ cells (Fig. 5B). Importantly, the KIF17b phosphorylation activity present in testis extracts was also blocked when H-89 was added to the reaction, stressing the importance of the PKA pathway in the phosphorylation of KIF17b in vivo (Fig. 5B).

**PKA Phosphorylates KIF17b in Vitro**—The down-regulation of KIF17b phosphorylation by the PKA inhibitor H-89 suggests that PKA can directly phosphorylate KIF17b. Another possibility is that PKA is a part of a phosphorylation cascade finally...
PKA-mediated Control of Kinesin KIF17b in Testis

Kinesins are classically known for their motor function and ability to transport cargos along the intracellular microtubule network in an ATP-dependent manner (18, 20). Kinesins move toward the plus end of the microtubules in contrast to another group of motor proteins, the dyneins, which are minus-end-directed microtubule motors. The discovery of KIF17b as a novel interaction partner for a transcriptional coactivator (ACT) and the ability of this kinesin to transport ACT from the active site of transcription into the cytoplasm has expanded the array of possible functions of kinesins by demonstrating that KIF17b can be directly involved in the regulation of transcription (13). Here we show that this function is neither dependent on the binding of KIF17b to microtubules nor the ATP-dependent motor function, thus demonstrating a novel microtubule-independent mechanism for KIF17b.

Even though the transport of ACT by KIF17b is not dependent on microtubules, KIF17b might have other functions in male germ cells, those mediated through microtubule binding. Indeed, KIF17b expression in male germ cells is not restricted on microtubules, KIF17b might have other functions in a structure consisting of microtubules and thought to be involved in the shaping of sperm nucleus (21) and in the principal piece of the sperm tail (12). Thus, it appears that, after functioning in a microtubule-independent manner in transporting ACT from nucleus to cytoplasm, KIF17b might switch to the microtubule-dependent mode and continue by functioning as a more “traditional” kinesin. This is very likely, because both the manchette and the sperm tail consist of highly organized microtubular arrays. The cargos associated with KIF17b during these stages have not been identified yet.

**FIG. 4.** KIF17b is phosphorylated in cells. A, in vivo phosphorylation of the Myc-tagged KIF17b overexpressed in COS-1 cells. 24 h after transfection, the medium was changed to the phosphate-deprived one. 36 h after transfection, cells were incubated for 4 h with 32P-labeled orthophosphate. After collection and lysis of the cells, immunoprecipitation was performed using the monoclonal anti-Myc antibody. The samples were run into a SDS-polyacrylamide gel, and phosphorylation was detected by autoradiography after drying the gel. A fraction of the samples was immunoblotted with anti-Myc antibody to confirm the expression of KIF17b. B, schematic representation of KIF17b deletion mutants used in the phosphorylation experiments. The motor domain, the traditional cargo-binding domain, and the ACT-binding region (black) are indicated. C, phosphorylation of KIF17b deletion mutants. Full-length Myc-KIF17b, Myc-KIF17b-(320–1038), Myc-KIF17b-(420–720), and Myc-KIF17b-(720–1038) were expressed in COS-1 cells, and the in vivo phosphorylation experiments were performed as in A. WB, Western blot.

**FIG. 5.** Protein kinase A mediates the phosphorylation of KIF17b. A, H-89 blocks phosphorylation of KIF17b in cells. COS-1 cells overexpressing Myc-KIF17b were treated with different signaling pathway inhibitors (H-89, 10 μM; Ro, Ro-31–8220, 5 μM; PD, PD98059, 20 μM; SB, SB203580, 20 μM; Toxin B, 10 mg/ml) in the presence of 32P orthophosphate. After incubation, the cells were lysed and immunoprecipitated using anti-Myc antibody. Phosphorylation was detected by autoradiography after drying the gel. Western blot (WB) was performed to show an equal amount of KIF17b in each lane. B, KIF17b is phosphorylated by testis kinases, and the phosphorylation is blocked by H-89. Myc-tagged KIF17b, immunoprecipitated from COS-1 cells, was incubated with 5 μg of the testis extract, [γ-32P]ATP, and 10 μM H-89, as indicated, in a buffer containing 50 mM HEPES, 100 mM KCl, 5 mM MgCl2, 0.5% Nonidet P-40, 5 mM NaF, 1 mM diethiothreitol, and 50 μM cold ATP at 37 °C for 20 min. The phosphorylation was detected by autoradiography of the dried SDS-polyacrylamide gel. Equal amounts of KIF17b were confirmed by immunoblotting with anti-Myc antibody.

**DISCUSSION**

Kinesins are classically known for their motor function and ability to transport cargos along the intracellular microtubule network in an ATP-dependent manner (18, 20). Kinesins move toward the plus end of the microtubules in contrast to another

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1. N. Kotaja, and P. Sassone-Corsi, unpublished observations.
PKA-mediated Control of Kinesin KIF17b in Testis

We further characterized the interaction between KIF17b and ACT and demonstrated that it is not a specific LIM domain in ACT but instead the integrity of the LIM domains that is needed for the interaction with KIF17b. This suggests that the general three-dimensional structure of ACT generates the interaction surface. The binding of KIF17b to ACT is not mediated by the C-terminal highly variable region of KIF17b that would correspond to the classical cargo-binding region in many different kinesins (19). On the contrary, ACT binds to the central stalk region of KIF17b, a domain generally thought to be a regulatory region, controlling processes such as kinesin oligomerization (18). The binding of ACT to the central stalk region, rather than the C-terminal region, suggests a regulatory role of ACT on KIF17b function. Because the structure of ACT consists only of the LIM domains, known to function as protein-protein interaction surfaces (17), it is tempting to speculate that ACT might function as a mediator of interactions between the kinesin and other proteins. Thus, it is possible that ACT is not only transported by KIF17b to the cytoplasm but could also have a more active role in the KIF17b function. Further possible members of this KIF17b-ACT complex remain to be characterized in the future.

Both ACT and KIF17b are expressed in round spermatids, where ACT is always nuclear and KIF17b is shuttling between the nucleus and the cytoplasm. Only in stage IX spermatids, when CREM-dependent transcription ceases and the spermatid nucleus starts to elongate, does ACT relocate to the cytoplasm together with KIF17b (13). This movement has to be strictly regulated to avoid premature relocalization of ACT. In this study, we have clarified the signals regulating KIF17b localization by showing that cyclic AMP-dependent PKA controls the subcellular localization and phosphorylation of KIF17b. KIF17b is expressed also in other stages of germ cell development, but the only developmental phase where KIF17b is shuttling between the nucleus and cytoplasm is in round spermatids, when it transports ACT from the nucleus to the cytoplasm. Because the inhibition of PKA signaling disturbs the nuclear-cytoplasmic shuttling of KIF17b, it is likely that the regulation of KIF17b by PKA pathway occurs at this stage of germ cell development.

We have found that KIF17b is a phosphoprotein (Fig. 4). It is likely that various protein kinases can phosphorylate KIF17b at many separate sites, but among all the tested signaling pathway inhibitors, PKA inhibitor H-89 was the only drug able to block the phosphorylation in cultured cells. This suggests that PKA is an important regulator of KIF17b phosphorylation. As shown by in vitro phosphorylation assay using the PKA catalytic subunit, KIF17b is a substrate of PKA. However, it is unclear whether KIF17b is phosphorylated directly by PKA in vivo or whether the phosphorylation is mediated by other kinases as a result of signal transduction cascades. KIF17b is heavily phosphorylated, which makes the study of specific phosphorylation sites more difficult. KIF17b contains three consensus PKA sites in its C-terminal region, but mutation of these sites does not abolish its phosphorylation levels in cultured cells. This indicates that other yet unidentified sites can be targets for phosphorylation.

The role of PKA in KIF17b regulation may include nuclear import, export, and cytoplasmic retention. The main nuclear localization signals are located in the C terminus of KIF17b, because the fragment of KIF17b containing only the C terminus is localized predominantly in the nucleus, and the KIF17b deletion lacking the C terminus is exclusively cytoplasmic. The inhibition of PKA activity changes the localization of KIF17b from only cytoplasmic to nucleocytoplasmic. Therefore, it is likely that the nuclear import is regulated by phosphorylation, and the lack of phosphorylation induces the transport of KIF17b into the nucleus. Another possibility is that the PKA pathway controls the cytoplasmic retention of KIF17b (for example, the binding of KIF17b to specific cytoplasmic structures). When the pathway is blocked, KIF17b starts leaking to the nucleus, thus causing the diffuse localization pattern. The export of KIF17b from the nucleus to the cytoplasm is known to be controlled by the chromosomal region maintenance 1 nuclear export pathway (13). Thus, a third possibility is that PKA-mediated phosphorylation stimulates the nuclear export, and in the absence of PKA signaling, the newly synthesized

**Fig. 6.** KIF17b can be directly phosphorylated by PKA. A, PKA phosphorylates KIF17b in vitro. Myc-tagged KIF17b was immunoprecipitated from COS-1 cells and incubated with [γ-32P]ATP and the catalytic subunit of PKA as indicated. The conditions were the same as shown in Fig. 5C. B, phosphorylation of the C-terminal region of KIF17b containing the three potential PKA sites is down-regulated by H-89. Myc-KIF17b(720–1038) was transfected into COS-1 cells, and in vivo phosphorylation was performed as described in the legend to Fig. 5A. Equal amounts of the mutant in each reaction were confirmed by Western blotting (WB) with anti-Myc antibody. C, the three putative PKA sites in the C terminus of KIF17b are not required for the phosphorylation in cells. In vivo phosphorylation was performed as described in the legend to Fig. 5A to compare phosphorylation of the Myc-tagged KIF17b mutant with serines 729, 893, and 931 mutated to alanines (S729A,S893A,S931A) to phosphorylation of the wild type KIF17b (WT).

**Fig. 7.** Mutation of the potential PKA sites in the C terminus of KIF17b affects the subcellular localization of KIF17b. COS-1 cells transfected with wild type KIF17b or mutant KIF17b with serines 729, 893, and 931 mutated to alanines (KIF17b/S729A,S893A,S931A) and ACT were incubated with 10 μM H-89 as indicated; immunofluorescence was performed using the anti-KIF17b antibody. Cells expressing KIF17b in the nucleus, cytoplasm, or in both compartments were counted. The values represent means ± S.D. from three independent experiments.
KIF17b is imported into the nucleus but cannot be efficiently exported anymore to the cytoplasm.

The PKA holoenzyme consists of two catalytic subunits and two regulatory subunits. The three different isoforms of somatic catalytic subunits are Ca, Cβ, and Cy. Ca and Cβ are ubiquitously expressed in most tissues, whereas Cy is a transcribed transposon that is expressed only in primate testis (22).

Interestingly, Ca generates a sperm-specific isoform, Cs, a product of an alternative transcript of the Ca gene (23, 24, 25). Both Ca and Cs are expressed during spermatogenesis, but their expression is mutually exclusive (24). Ca is expressed in somatic cells, spermatagonia, and preleptotene spermatocytes, whereas Cs is germ cell-specific; it first appears in mid-pachytene spermatocytes, and the expression continues in round and elongating spermatids and finally in the tail of mature sperm (24). Thus, the expression of KIF17b and the Cs catalytic subunit of PKA overlaps in round and elongating spermatids when KIF17b is active in shuttling between the nucleus and the cytoplasm, indicating that the Ca catalytic subunit is involved in the phosphorylation of KIF17b at this stage of spermatogenesis. PKA is activated by cyclic AMP produced by adenylyl cyclase (AC). In testis, at least two categories of ACs have been described, the membrane-associated ACs that are regulated by G protein-associated receptors and the soluble ACs that are modulated by bicarbonate (26). Both the membrane-bound and soluble AC isoforms are expressed in postmeiotic round spermatids (27), where KIF17b and ACT are active.

FSH is a pituitary hormone regulating sperm development through its adenylyl cyclase-coupled receptors located at the cell surface of somatic Sertoli cells in the seminiferous epithelium. Sertoli cells are in close contact with germ cells and constantly communicate with these cells and regulate their functions through paracrine signaling and junctions between the Sertoli and germ cells (14). The communication between Sertoli cells and germ cells is not well characterized. Although no adenylyl cyclase-coupled FSH receptors have been found on germ cells, PKA activity changes during germ cell differentiation, to reach a maximum level in spermatids (28). cAMP is thought to play an important role in governing the timing of postmeiotic gene activation in response to FSH signaling (29). FSH-stimulated cAMP production is stage-dependent and is particularly significant at stages I–V (30). During these same stages, KIF17b shuttles between the nucleus and the cytoplasm (13), suggesting that this increase in cAMP production could be involved in the regulation of KIF17b movements.

Together these results demonstrate a novel, microtubule-independent function for a kinesin motor protein that is regulated by PKA and cAMP, thus linking PKA signaling to the control of CREM-dependent transcription in postmeiotic germ cells. Because ACT is able to enhance CREM transcription in a phosphorylation-independent manner (11), it appears that KIF17b and its regulation by PKA offer a new model for cAMP responsiveness of germ cells without the direct phosphorylation of CREM.

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