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Tctex2-Related Outer Arm Dynein Light Chain Is Phosphorylated at Activation of Sperm Motility

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When the motility of sperm is activated, only one light chain of flagellar outer arm dynein is phosphorylated in many organisms. We show here that the light chain to be phosphorylated was shown to be light chain 2 (LC2) in rainbow trout and chum salmon sperm and LC1 in sea urchin sperm. Molecular analyses of the phosphorylated light chains from sperm flagella of the salmonid fishes and sea urchin revealed that the light chains are homologs of the mouse *t* complex-encoded protein Tctex2, which is one of the putative *t* complex distorters. These results suggest that mouse Tctex2 might also be a light chain of flagellar outer arm dynein and that the abortive phosphorylation of Tctex2/outer arm dynein light chain might be related to the less progressive movement of sperm.

Transmission ratio distortion (TRD) or meiotic drive in *t*-bearing mice is the most dramatic example of non-Mendelian transmission: +/*t* heterozygous males transmit the *t*-bearing copy to more than 95% of their progeny of the chromosome. TRD in the +/*t* male is considered to be related to the dysfunction of +-sperm, which somehow leads to the transmission in favor of *t*-sperm (1, 2). This phenomenon has been extensively studied by genetical approaches: these studies have proposed that TRD is due to the interaction of at least four distorters (Tcd1-4) with a common target, the responder (Tcr) (3, 4). Differential screening of cDNA library from wild type testis with those from wild-type or *t*/*t* testis has identified a series of *t* complex testis-expressed proteins, Tctex proteins, which are related to TRD or male sterility in *t*-bearing mouse. Of these, Tctex1 (Tcd1) has recently been identified as a light chain (LC) of cytoplasmic dynein (5), an inner arm dynein of *Chlamydomonas* (6) or an outer arm dynein of sea urchin sperm (7). Tctex2 (Tcd3), on the other hand, was identified as one of the LCs of axonemal outer dyneins from both *Chlamydomonas* (8) and sea urchin sperm flagella (DDBJ/EMBL/GenBank Database accession number AB010055).

A dynein-mediated TRD model has been proposed to the mechanism for how the +-sperm become defective during spermatogenesis in +/*t* males (8). Two important events might be involved in the mechanism for TRD, if we assume that the +/*t* mouse produces equal numbers of +-sperm and *t*-sperm, where only *t*-sperm show progressive motility and have advantages over +-sperm in fertilization. The first event is the process in which two types of sperm with different motility are produced by the action of Tcr. The second important event is that +-sperm become defective in motility by the action of defective Tcds. Since two out of the multiple Tcds have been shown to be related to dynein, it is likely that the immotility of +-sperm is due to the dysfunction of dynein. However, it has not been concluded how the mutations of Tcds connect to the defective sperm motility.

cAMP-dependent phosphorylation of sperm axonemal components is the most important intracellular trigger for causing the initiation, activation, hyperactivation of sperm motility in salmonid fish (9), sea urchin (10, 11) mussel (12), tunicate (13) and mammals (14, 15). The major target of cAMP-dependent protein phosphorylation is a structural component of dynein (12, 16–19). Although outer arm dynein generally contains six to eight LCs, a few, or in most cases only one LC can be phosphorylated by a cAMP-dependent protein kinase. The dynein LCs so far known to be phosphorylated in a cAMP-dependent manner include a

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank database under Accession No. AB015761.

Abbreviations used: LC, light chain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; TRD, transmission ratio distorsion.

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Paramecium ciliary 29 kDa LC (16), a 20-kDa LC in *Spisula* gill cilia (12) and a 18-20 kDa LC in *Ciona* sperm (18).

Recently we have shown that the 22 kDa outer arm dynein LC in salmonid fish is phosphorylated in a cAMP-dependent manner in parallel to the activation of sperm motility (19). In this paper, we show by molecular cloning of this protein that it is highly homologous to Tctex2. To explore our finding in distantly related species, we have identified a Tctex2-related LC of outer arm dynein from sea urchin sperm as the only LC to be phosphorylated. Thus we suggest that LCs of outer arm dynein to be phosphorylated at the activation of sperm motility is Tctex2-related proteins and that murine Tctex2 might be also a light chain of outer arm dynein in sperm flagella.

MATERIALS AND METHODS

Phosphorylation of axonemal proteins and isolation of phosphorylated dynein. Two species of salmonid fishes (chum salmon *Oncorhynchus keta* and rainbow trout *Oncorhynchus mykiss*) and sea urchins (*Pseudocentrotus depressus* and *Anthocidaris crassispina*) were used in the present study. Sperm flagella and axonemes were prepared as described previously (20, 21). In the case of sea urchin, isolated flagella were kept on ice for 1 hr before use to lower the background level of the phosphorylated axonemal proteins by endogenous phosphatases.

Axonemes were diluted with a buffer containing 0.15 M KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris–HCl, pH 8.0, 1mM DTT, to a protein concentration of 2–3 mg/ml. One μl of [γ-^{32}P]ATP (diluted with cold ATP to 3.7 GBq/mmol; ATP concentration, 40 mM) was added to 40 μl of axoneme suspension and incubated for 10 min at 25°C in the presence or absence of 10 μM cAMP. Reactions were terminated by the addition of concentrated sample buffer for SDS–PAGE.

Axonemes were phosphorylated in the presence of 10 μM cAMP and 1 μM okadaic acid. After 0.6 M KCl or 0.5 M NaCl extraction, outer arm dynein was purified by 5–20% sucrose density gradient centrifugation.

Direct amino acid sequencing of salmonid LC2. In the case of chum salmon, the outer arm dynein was further dialyzed overnight against a low ionic strength buffer containing 5 mM Tris–HCl, pH 8.0, 0.1 mM EDTA and 0.1 mM DTT. The content was centrifuged through 5–20% sucrose density gradient and the LC2 containing complex was collected at 10S fraction. Concentrated 10S fraction was subjected to 12% SDS–PAGE, followed by staining with Coomassie brilliant blue. The region corresponding to LC2 was cut out, incubated with 500 μl of the sample buffer for SDS–PAGE for 10 min at room temperature under slow agitation, and subjected to peptide mapping with V8 protease by the method of Cleveland *et al.* (22). Proteolytic fragments were separated by SDS–PAGE and transferred to Immobilon-P^{seq} membrane (Millipore, Woburn, MA). The peptide sequences were determined by an automated protein sequencer (model 492, Applied Biosystem, Foster City, CA).

Oligonucleotides. Following primers were used in the present work: DLC22-A, 5′-CGC GGA ATT CAC (G/A/C/T)AT GGA (T/C)AT (A/T/C)GA (A/G)AT GCA-3′ (additive restriction site plus GC clamp is underlined); DLC22-C, 5′-CGC GGA ATT CGA (T/C)GA (T/C)GA (T/C)(A/T)(G/C) (G/A/C/T)AC A(T/C)C A-3′; DLC22-F, 5′-CGC GGG ATC CCA (G/A)TT (G/A)TC (G/A)TG (G/A)TC (G/A)TA-3′; DLC22-H, 5′-CGC GGG ATC CTC (G/A/C/T)C(G/T) (G/A)CA (G/A)TT (G/A)TC (G/A)T-3′; oligo-157, 5′-TGT GAA ACG AGG GTC TTC AGT-3′ (corresponding to nt 185-205 of a final LC2 clone); oligo-158, 5′-CTG CCT TTC ACA TTG CCC ATT-3′ (corresponding to nt 583–603 of a final LC2 clone); oligo-162, 5′-CTC TTT GCA GAA CGC CAG GAC-3′ (corresponding to nt 381-401 of a final LC2 clone); oligo-73, 5′-AAC TGG AAG AAT TCG CGG CCG CAG GAA G(T)18 (G/A/C)(G/A/C/T)-3′ (bifunctional primer contained in a First Strand cDNA Synthesis kit, Pharmacia).

Poly(A)$^+$ RNA from testis of rainbow trout. The messages for axonemal proteins were contained mostly in the testis at midstage from rainbow trout (23). mRNA was prepared from the testis one to two month before spawning by a guanidium isothiocyanate method, using QuickPrep mRNA purification kit (Pharmacia LKB, Uppsala, Sweden).

RT-PCR. First strand cDNA was synthesized from trout testis poly(A)$^+$ RNA using a First Strand cDNA Synthesis kit (Pharmacia LKB, Uppsala, Sweden). An aliquot of cDNA product was PCR-amplified with respect to specific cDNA target sequences using DLC22-A and DLC22-H which may flank the cDNA region of interest. PCR was carried out using an Elongase Enzyme mix (Gibco BRL, Gaithersgurg, MD) in a GeneAmp PCR System 2400 (Perkin–Elmer, Norwalk, CT). Amplification was started with heating at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 45°C for 2 min, and extension at 68°C for 2 min. Post-extension was at 68 °C for 10 min. An aliquot of first PCR product was again amplified using the nested primers, DLC22-C and DLC22-F. About 200-bp band was purified by electrophoresis with a low-melting agarose gel, subcloned into pBluescript II SK+ vector, and sequenced by Model 310 sequencer (Applied Biosystem, Foster City, CA).

3′- and 5′-RACE. Based on the sequences of PCR products described above, 3′- and 5′-RACEs were performed to determine the whole coding region of trout LC2 cDNA. For 3′-RACE, an aliquot of the first strand cDNA product was PCR-amplified using two primers (DLC22-A and oligo-73). Whole product was purified by a Qiagen PCR purification kit (Qiagen K.K., Tokyo, Japan). An aliquot was again PCR-amplified using a gene-specific primer (oligo-157) and oligo-73. PCR product was ligated into a T-vector. Four clones were sequenced. They contain nt 157 to 515 of a final clone (Fig. 1A). The 5′-end of trout LC2 cDNA was obtained by a Marathon cDNA Amplification kit (Clonetech Inc., Palo Alto, CA) using gene-specific two nested primers (oligo-158 and -162). PCR product was ligated into a T-vector. Five clones were sequenced. They contain nt 1 to 162 of a final clone.

RESULTS

A certain proteins in the demembranated model of chum salmon sperm are phosphorylated in the presence of [^{32}P]ATP alone. Phosphorylation of two proteins with molecular masses of 22 and 15 kDa were significantly increased by the addition of cAMP (Fig. 1A). The former has been recently identified as a LC of outer arm dynein (19) and the latter was shown to be phosphorylated at its tyrosine residue and exist as an complex with other proteins at the base of sperm flagella (24, 25). The outer arm dynein of salmonid fish sperm contains six light chains and only the 22-kDa light chain (LC2) can be phosphorylated (Fig. 1C; also see 19). Increase in phosphorylation by cAMP was observed in LC2 in isolated flagellar axonemes (Fig. 1B), demonstrating that this phosphorylation is thought to

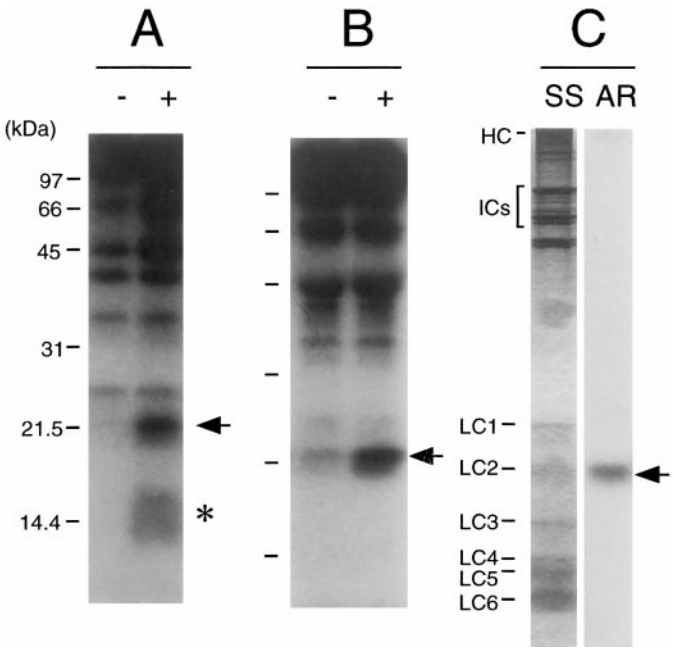

FIG. 1. cAMP-dependent phosphorylation of axoneme proteins in chum salmon. (A) Phosphorylation of the proteins in demembranated sperm in the absence (−) or presence (+) of cAMP. (B) Phosphorylation of the proteins in isolated flagellar axoneme in the absence (−) or presence (+) of cAMP. (C) Silver staining (SS) and the corresponding autoradiogram of the fraction of outer arm dynein collected from sucrose density gradient. Arrows indicate the 22-kDa dynein light chain (LC2). The asterisk shows a 15-kDa protein which is also phosphorylated in a cAMP-dependent manner but is present only in demembranated sperm. Molecular mass markers are given in kDa and the positions are indicated on the left of the gels.

be closely related to cAMP-dependent activation over the flagellar axoneme. The increase in phosphorylation was also observed in a 48-kDa band which is probably the regulatory subunit of a cAMP-dependent protein kinase (19).

In contrast to salmonid fish, the incorporation of ^{32}P into demembranated axonemal proteins was very low in the case of sea urchin, probably because spontaneous phosphorylation of axonemal proteins by a cAMP-dependent protein kinase had already occurred by dilution of sperm into sea water or by demembranation of sperm flagella with Triton X-100. To achieve high ^{32}P-labeling in proteins, the isolated sperm flagella were kept on ice for 1 hr before demembranation. The ^{32}P-labeled axonemes were treated with a solution containing 0.6 M KCl to extract outer arm dynein and the extract was centrifuged through a sucrose density gradient (Fig. 2A). A ^{32}P-incorporated 23-kDa protein was found to sediment at fractions 6-8 with highest density at fraction 7 (Fig. 2A, inset). The band density in the autoradiogram agreed with the ATPase activity pattern of outer arm dynein. As reported previously (7, 28, 29), sea urchin outer arm dynein contains six LCs in addition to intermediate chains (ICs) and heavy chains (HCs). Protein staining and corresponding autoradiography of the outer arm dynein (fractions 6–8) clearly showed that phosphorylated protein is LC1 (Fig. 2B).

The phosphorylation of LC1 occurred even in the absence of cAMP (data not shown), probably because cAMP-dependent protein kinase had been already activated when sperm were diluted in sea water or by demembranation of sperm flagella with Triton X-100. The phosphorylation, however, was inhibited by a specific inhibitor for cAMP-dependent protein kinase, H89, at more than 50 μM (data not shown).

For molecular cloning of trout LC2, direct amino acid sequencing was performed using LC2 purified from chum salmon sperm rather than rainbow trout, because a large amount of sperm can be easily obtained from the salmon. Since the N-terminus of LC2 was blocked, internal sequences were determined by the direct sequencing of V8-cleaved peptides. We could obtain sequence 1, STMDIEMHDDDDSHQQKR, and sequence 2, XXNEAFXELAYDHDNCRDVADKVAADV-LAFXKEQVFDRYRYVARVVVXEXK where X is unidentified amino acid residue. Based on these sequences, primers were synthesized for RT-PCR: two nested primers, DLC22-A and DLC22-C, correspond to sequences TMDIEMH and DDDSHQ of sequence 1, respectively, and complementary, two nested primers, DLC22-G and DLC22-F, correspond to sequences HDNCRD and YDHDNC of sequence 2, respectively.

RT-PCR product contained nt 160 to 356 of a final clone. Due to the species specificity between chum salmon and rainbow trout, deduced amino acid did not completely coincide with those determined by the direct sequencing. The 3′- and 5′-RACEs were performed to determine the whole coding region of trout LC2 cDNA (DDBJ/EMBL/GenBank Database, accession number AB015761). The trout cDNA clone encodes 169 amino acids. Calculated molecular weight and pI are 19,520 and 9.05, respectively.

PSI-BLAST search (26) revealed that trout LC2 is a homolog of outer arm dynein LC1 from sea urchin (E-value 75), murine *t*-complex testis-specific protein 2 (Tctex2) (E-value 71)(27), and 19 kDa outer arm dynein LC from *Chlamydomonas reinhardtii* (E-value 55)(8). Multiple sequence alignment of trout LC2, sea urchin LC1, *Chlamydomonas* 19 kDa protein, and mouse Tctex2 protein generated by the program CLUSTAL W is shown in Fig. 3A. The PLOTSIMILARITY program which visualizes the conservative region of the aligned sequences showed that the similarity is limited to the C-terminal regions and there are no significant similarities in the N-terminal regions of these proteins (Fig. 3B).

Since trout LC2 is a substrate of cAMP-dependent protein kinase, we searched whether Tctex2-related proteins contain the phosphorylation sites in the se-

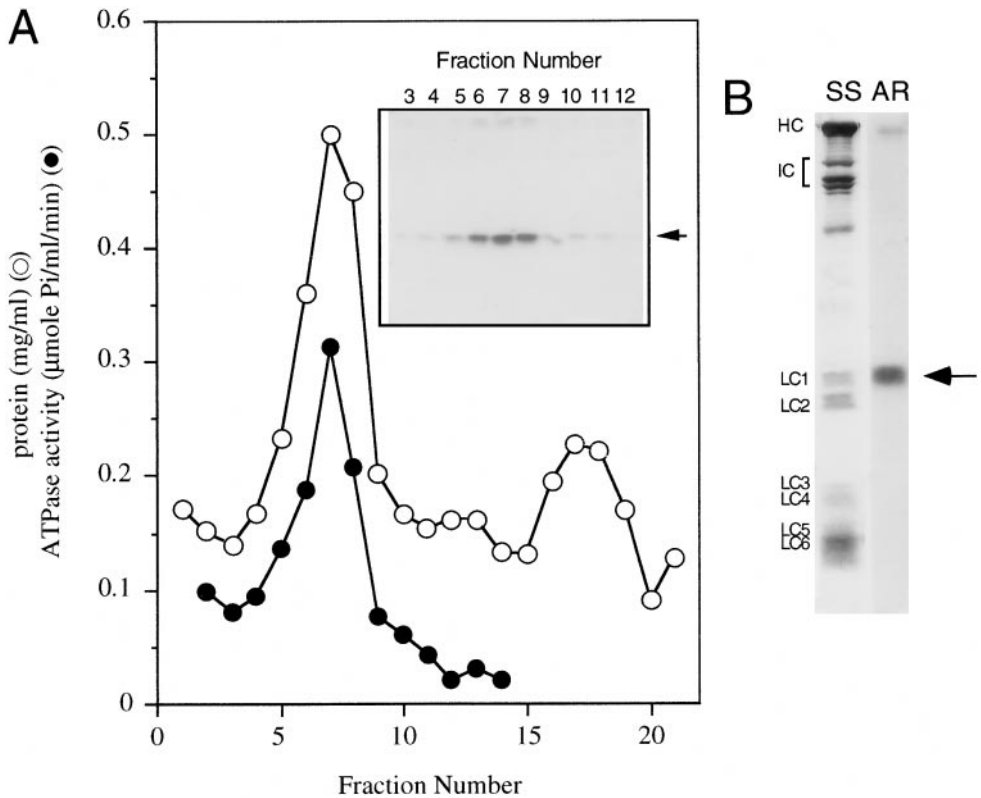

FIG. 2. Phosphorylation of outer arm dynein LC in sea urchin sperm flagella. (A) Sedimentation profile of proteins and ATPase activity of 0.6 M KCl extract from flagellar axonemes through sucrose density gradient. Inset, autoradiogram of phosphoproteins in each fraction after separation by SDS–PAGE (12% gel). (B) Analysis of outer arm dynein by silver staining (*SS*) and corresponding autoradiogram (*AR*). Arrows show the phosphorylated 23-kDa dynein light chain (LC1).

quences. Consensus sequence for possible phosphorylation sites by cAMP-dependent protein kinase is [Arg/Lys]-[Arg/Lys]-X-[Ser/Thr]. Trout LC2 has three such sites at amino acid residues 4–7 (RRGS), 13–16 (RKDS), and 40–43 (KRGS) (Fig. 3A), which agrees with the previous result showing that the phosphoamino acid residue is Ser (19). Sea urchin LC1 has one cAMP-dependent phosphorylation site (RRAT) located near the N-terminus (amino acid residues 3–6). Mouse Tctex2 contains one site (RKPS) at amino acid residues 25–28. These sites were all located within the N-terminal one fourth of the entire length. However, no consensus sequence for cAMP-dependent phosphorylation site was found in the *Chlamydomonas* 19 kDa protein (Fig. 3A).

DISCUSSION

In the present paper, we have presented the first evidence suggesting that a Tctex2-related dynein LC is phosphorylated at activation of sperm motility. In salmonid fish sperm, LC2 appears to be phosphorylated by a cAMP-dependent protein kinase (Fig. 1). In sea urchin sperm, no direct evidence was obtained here to show that LC1 is phosphorylated by a cAMP-dependent protein kinase. Several lines of evidence however suggest that sea urchin LC1 is phosphorylated by a cAMP-dependent protein kinase. First, several phosphoproteins in sea urchin sperm flagella have been critically reported in relation to the activation of sperm motility (11) and cAMP apparently activates sperm motility by cAMP-dependent protein kinase responsible for the phosphorylation of sperm axonemal proteins (10), probably through type II cAMP-dependent protein kinase (30). Second, activation of sperm motility accompanies cAMP-dependent phosphorylation of the light chain(s) of outer arm dynein in many species so far reported (9, 12, 18). Third, although we could observe only slight difference in the phosphorylation of LC1 between the conditions in the absence and presence of cAMP, an inhibitor specific to cAMP-dependent protein kinase, H89, significantly decreased the phosphorylation level of LC1 at 50 μM.

From the sequence analysis of trout LC2, the phosphorylation site for cAMP-dependent protein kinase was identified at the extreme N-terminal region of the molecule. Corresponding sites were found in murine Tctex2 and sea urchin outer arm dynein LC1, both also

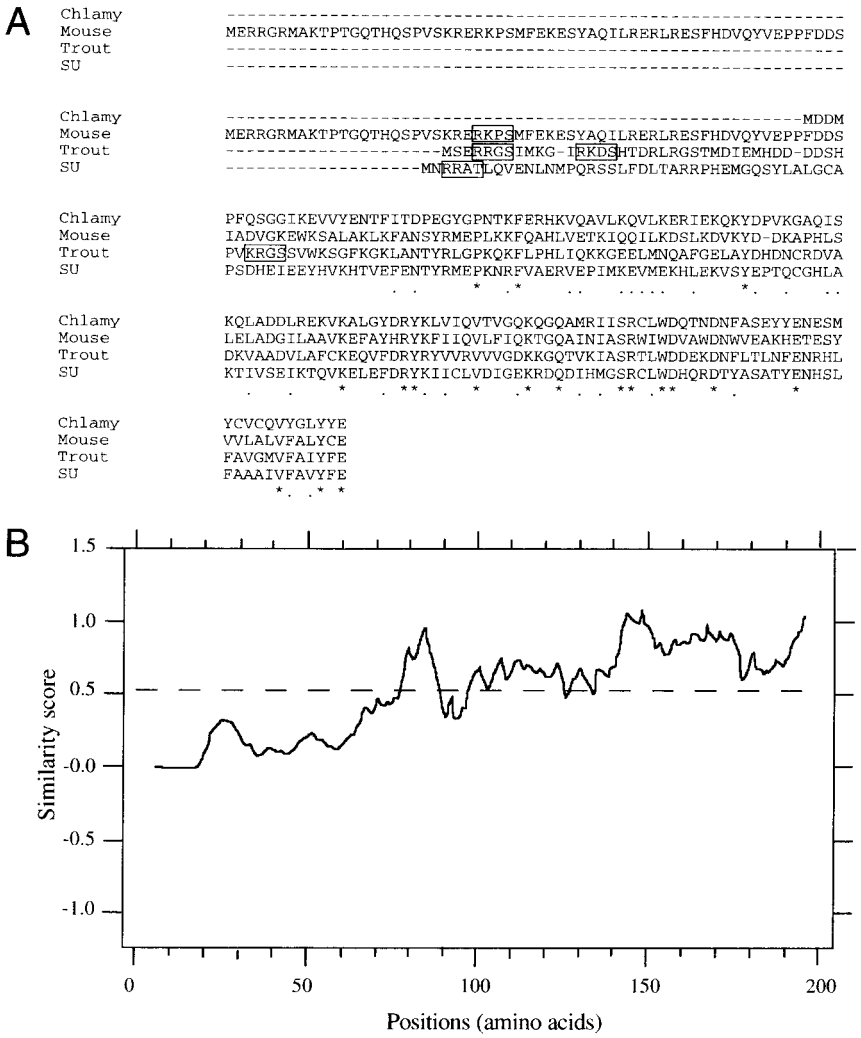

FIG. 3. Comparison of Tctex2-related dynein light chains. (A) Multiple alignment of Tctex2-related dynein light chains by CLUSTAL W. *Chlamy, Chlamydomonas* 19-kDa dynein LC; *Mouse,* mouse Tctex2; *Trout,* trout LC2; *SU,* sea urchin LC1. Asterisks indicate identical amino acids in all sequences. Dots indicate conservative replacements. The motif sequences for phosphorylation by cAMP-dependnet protein kinase are boxed. (B) A plot showing the similarity among four sequences by PLOTSIMILARITY.

at the N-terminal regions. However, no such site was found in the 19-kDa *Chlamydomonas* outer arm dynein LC. This coincides with the previous observation that while trout outer arm dynein LCs were phosphorylated by cAMP-dependent protein kinase, any LCs of outer arm dynein in *Chlamydomonas* was not phosphorylated (31, 32). Rather cAMP gives inhibitory effects on the flagellar motility in *Chlamydomonas* (33).

The importance of phosphorylation of outer arm dynein LC in sperm flagellar motility or ciliary motility has been described in many organism. In salmonid fish, the phosphorylation of outer arm dynein LC by cAMP-dependent protein kinase might be related to the activation of sperm motility (19). In *Paramecium* cilia, the phosphorylation of 29-kDa dynein LC apparently increases the sliding velocity of microtubules (16), suggesting the participation of the phosphorylation of dynein LC in the activation of dynein-driven microtubule sliding.

The present paper also sheds light on the mechanism of TRD, because the function of Tctex2 was first inferred in terms of axonemal motility. The +/*t* mouse produces an equal amount of +-sperm and *t*-sperm. The putative function of Tcr is considered to incorporate Tcds into axonemes during spermatogenesis. It is proposed that normal form of Tcr (Tcr+) could incorporate both normal and dysfunctional Tcds, whereas defective form of Tcr (Tcr*t*) could incorporate only normal Tcds (8). The present result suggests that sperm containing dysfunctional Tctex2 has a defect in cAMP-dependent phosphorylation of an outer arm dynein light chain, which may

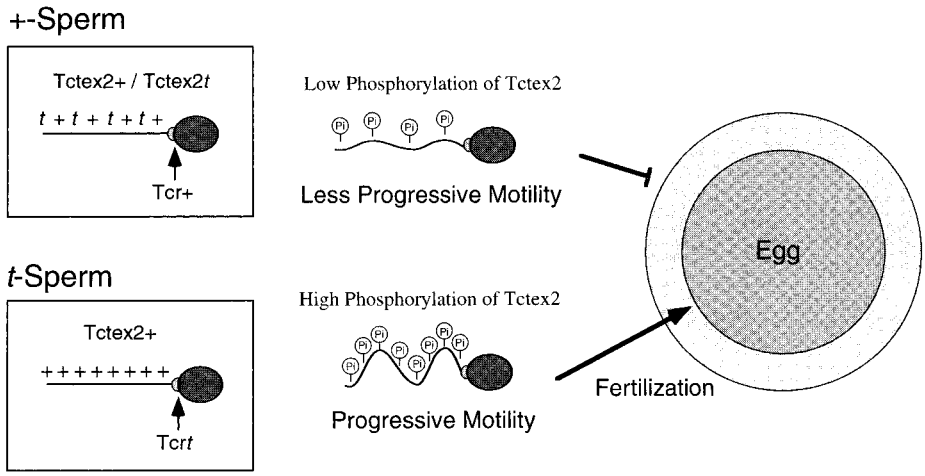

FIG. 4. A model for TRD mediated by the phosphorylation of dynein light chain/Tctex2. The +/*t* mouse produces equally the +-sperm and *t*-sperm. Tcr is hypothesized as a gatekeeper in determining of Tcds to be assembled into the axoneme (8). In +-sperm with wild type Tcr (Tcr+), both normal Tctex2 (Tctex2+) and defective Tctex2 (Tctex2*t*) might be incorporated into the axoneme. This would lead to a low number of phosphorylated Tctex2 and less progressive motility of sperm. In *t*-sperm with Tcr*t*, however, only Tctex2+ might be incorporated into the axoneme. This would lead to a high number of phosphorylated Tctex2 and progressive motility of sperm. Only *t*-sperm with progressive motility might predominantly penetrate into egg and succeed in fertilization, resulting in TRD.

cause inefficient microtubule sliding, resulting in less progressive motility (Fig. 4).

Multiple Tcd genes have been identified as acting on a Tcr gene in connection to TRD (3, 4). So far, two out of at least four Tcds have been shown to be the components of outer arm dynein (7, 8; this study). It is widely accepted that the outer arm dynein functions not in fundamental mechanism for the formation and propagation of flagellar waves, but in elevating the frequency of flagellar beating (34, 35). In mammalian sperm, the significance of outer arm dynein has been implied in generating progressive motility, or hyperactivation, which is apparently necessary for sperm to penetrate into the egg (36, 37). Therefore, only *t*-sperm with normal Tctex2 would be successful in fertilization, resulting in TRD (Fig. 4).

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