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Original article

Immunological cross-reaction between sperm dynein heavy chains from different species

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Summary — The 19S outer arm dynein of trout sperm flagella is a complex of proteins composed of two heavy chains, five intermediate chains and at least six light chains. After dialysis against a low ionic strength buffer, its β subunit was purified and used to generate a rabbit polyclonal antibody. This polyclonal antibody reacted strongly with the trout β dynein heavy chain (DHC) but not with the trout α dynein heavy chain; it also recognised the dynein intermediate chains and tubulins. A specific antibody directed against the β DHC was obtained by blot-affinity purification. This specific anti-trout β DHC reacted with the β DHC from the sea-urchin sperm 21S dynein and also with one heavy chain (> 400 kDa) of demembranated ram sperm. This anti-β DHC antibody, and also the whole polyclonal, did not react with heavy chains in trout brain or liver extracts, sheep brain extract or purified brain and testicular cytoplasmic dyneins. These results suggest that a specific epitope of one of the sperm outer arm dynein heavy chains is conserved throughout evolution and that this epitope is not present on cytoplasmic dynein.

dynein-ATPase / antibody / spermatozoa / trout / sea urchin / ram / flagella

Résumé — Études des similarités immunologiques entre les chaînes de haut poids moléculaire de la dynéine-ATPase. *La dynéine de bras externe de l'axonème de spermatozoïde de truite est une particule formée de deux chaînes polypeptidiques (α et β) de haut poids moléculaire (430 et 415 kDa respectivement), de cinq chaînes de poids moléculaire moyen (entre 50 et 70 kDa), et six chaînes de petit poids moléculaire (> 30 kDa). Après dialyse contre un tampon à faible force ionique, cette particule génère une sous-unité contenant la chaîne β et certaines des chaînes intermédiaires. Cette particule après purification a servi à immuniser un lapin. L'anticorps polyclonal obtenu reconnaît la chaîne de haut poids moléculaire β, les chaînes intermédiaires et les tubulines. Un anticorps monospécifique contre la chaîne β a été obtenu par purification du polyclonal sur réplique de nitrocellulose. Cet anticorps reconnaît uniquement la chaîne β dans l'axonème de spermatozoïde de truite et réagit aussi avec la chaîne β de la particule 21 S de dynéine flagellaire de spermatozoïde d'oursin. Cet anticorps reconnaît aussi une chaîne de haut poids moléculaire (> 400 kDa) présente dans les spermatozoïdes de bélier démembranés. En revanche, ni cet anticorps purifié ni l'anticorps total ne reconnaissent des chaînes de haut poids moléculaire dans des extraits de tissus de foie ou de cerveau de truite. Il en est de même avec des extraits de testicule ou de cerveau de mouton, ou de la dynéine cytoplasmique obtenue à partir de ces deux tissus. Ces résultats démontrent que les chaînes α et β qui composent la*

dynéine de bras externe de l'axonème de spermatozoïde de truite sont deux isoformes immunologiquement distinctes. Ils montrent aussi que certain(s) épitope(s) spécifique(s) d'une isoforme de chaîne de haut poids moléculaire est maintenu au cours de l'évolution, et que ce ou ces épitopes sont uniquement flagellaires. Ce ou ces épitopes pourraient intervenir soit dans la génération du battement flagellaire soit dans l'assemblage des bras externes de dynéine.

dynéine-ATPase / anticorp / spermatozoïdes / truite / oursin / bélier / flagelle

INTRODUCTION

Dynein-ATPases are molecular complexes with very high molecular mass (> 1.3 MDa) involved in microtubule translocation during the beating cycle of the axoneme of cilia and flagella (for a review, see Witman et al, 1994). This class of enzyme is also involved in cytoplasmic organelle transport and some of the chromosomal movements during the cell cycle (Holzbaur et al, 1994). The biochemical properties, molecular composition and architecture, and also the organization of dynein-ATPases, were investigated using mainly axonemal outer arm dyneins from protists and invertebrates. The flagellar outer arm dynein–ATPase complex can be extracted from the axoneme by high ionic strength treatment. The dynein subunits are further purified by sucrose gradient centrifugation, where they sediment as large complexes between 10S to 30S. Each of the subunits contains at least one high molecular weight chain (> 400 kDa), that exhibits ATPase activity, associated with intermediate and low molecular weight chains (for reviews, see Witman, 1989, 1992; Witman et al, 1994).

Analysis of the outer arm dynein from trout sperm showed that this vertebrate axonemal dynein is very similar in its structure, properties and behaviour to the sea urchin outer arm dynein (Gatti et al, 1989; King et al, 1990). In this paper, antibodies against the trout sperm outer arm dynein were obtained and immunochemistry was used as a tool to search for similarities between the sperm dynein heavy chains from different species, and also to investigate the relation between axonemal and cytoplasmic dynein heavy chains.

MATERIALS AND METHODS

Collection of sperm

Trout sperm *(Oncorhynchus mykiss)* were obtained by dissecting the testis of ripe males averaging 2–3 kg in weight (50 mL at about 4 x 10^{10} sperm/mL). Aliquots of 5 mL were frozen in straws in liquid nitrogen until use.

Ram sperm (Île-de-France) were obtained by ejaculation using an artificial vagina (about 2 mL at 10^9 sperm/mL) and diluted with 20 mL of phosphate buffer saline solution (PBS: NaCl, 140 mM; KCl, 15 mM; Na_2HPO_4, 7 mM; KH_2PO_4 1.5 mM; $MgCl_2$, 0.5 mM; pH 7.5) containing glucose (5 mM) at 20 °C. The suspension was centrifuged (1500 *g*, 10 min), the supernatant discarded and the pellet resuspended in PBS. This operation was repeated three times and the last pellet was resuspended in 5 mL PBS.

Chemicals in the best available grade were from Prolabo (Paris, France) or Sigma (Saint-Quentin Fallavier, France).

Trout and sea urchin axonemal dynein purification

Isolation of trout sperm axonemes and purification of 19S dynein and its β subunit were achieved as previously described (Gatti et al, 1989; King et al, 1990; Moss et al, 1991). Sea urchin sperm *(Sphaerechinus granularis)* were obtained by the induction of spawning in artificial sea water by injection of 0.5 M KCl. Isolation of sea urchin sperm axonemes, and purification of the 21S dynein and its β/IC subunit were performed as previously described (Bell et al, 1982; Sale and Fox, 1988).

Antibodies

A rabbit was injected with the pooled fractions of a sucrose gradient containing the purified β subunit of the trout 19S dynein. Four injections, each of about 150 μg protein were given; the initial injection was with Freund's complete adjuvant and the three subsequent boosts were with incomplete adjuvant. The rabbit was bled before each injection and the serum tested by immunoblot against intact axonemes. No cross-reactivity was observed with the pre-immune serum.

Antibodies specific against the trout β DHC were obtained by nitrocellulose blot-affinity purification as described by Olmsted (1981). The pooled 19S fractions from the sucrose gradient were loaded along the entire length of a 4% SDS-polyacrylamide gel and, after migration, transferred on nitrocellulose. The heavy chains were localized using Ponceau S Red staining and then excised. After blocking in TBS-Tween with 5% goat serum (TBS: 20 mM Tris pH 7.3; 125 mM NaCl; Tween 20, 0.05% v/v) the strip was incubated with the rabbit serum (1:500) for 2–3 h at room temperature, washed in TBS-Tween 3 x 15 min, and the antibodies eluted from the strip with 0.8 mL glycine/EGTA (200 mM and 1 mM respectively) at pH 2.8. The solution was removed and neutralized with 100 μL Tris-base 1 M (pH 9.0), then 100 μL PBS (10 x) were added. Typically the complete cycle was performed twice with two strips of nitrocellulose and the final solution contained 50–80 μg/mL protein. The affinity purified antibodies were used after a fivefold dilution in TBS-Tween.

The mouse monoclonal anti-chick brain cytoplasmic dynein heavy chain (440 kDa) was obtained from Sigma (product no D-1667, clone 440.4; Steuer et al, 1990).

Preparation of cellular extracts and cytoplasmic dynein

Trout brains (2.5 g from five fish) and livers (about 100 g) were obtained from freshly killed trout, and used immediately or frozen in liquid nitrogen. Ewes brains and ram testis were obtained immediately after animal slaughter and plunged into cold PBS containing 2 mM *p*-aminobenzamidin. The brain dissection gave 125 g of white matter. One hundred grams of tissue from one testis was used. The different tissues were treated as described by Paschal et al (1991) to prepare the low speed extract (24 000 *g* supernatant of the crude homogenate of the tissue) and the cytosolic extracts (150 000 *g* supernatant of the low speed extract) and also to purify the cytoplasmic dynein.

Gel electrophoresis

Samples were analysed using either 6–16% acrylamide gradient or 4% acrylamide SDS-PAGE (8 x 6 cm gels) containing 2 M urea under reducing conditions. Gels were silver-stained to visualize the less intense bands.

In order to improve the visualization of the mammalian sperm high molecular weight proteins by reducing the amount of DNA that migrated into the gel when sperm were used directly, we extracted the protein from the sperm using a method modified from Baccetti et al (1980).

Protein blotting

Transfer of proteins to nitrocellulose (Hibond-C 0.45 μm, Amersham, Les Ulis, France) was achieved using a semi-dry transfer apparatus at 0.8 mA/cm^2 for 2 h. The second antibody was a goat anti-rabbit antibody conjugated to peroxidase (Pasteur Diagnostic, Paris, France) and diluted 1:5 000 in TBS-Tween. Visualization of the peroxidase on immunoblots was achieved by the 4α-chloronaphthol method.

RESULTS

Characterization of the anti-β dynein heavy chain antibody

The antigen consisted of the β subunit obtained by low ionic strength dialysis of the 19S outer arm dynein as previously described (King et al, 1989; Moss et al, 1991). This subunit was composed of the β heavy chain and a small amount of intermediate chains (fig 1) (King et al, 1989; Moss et al, 1991).

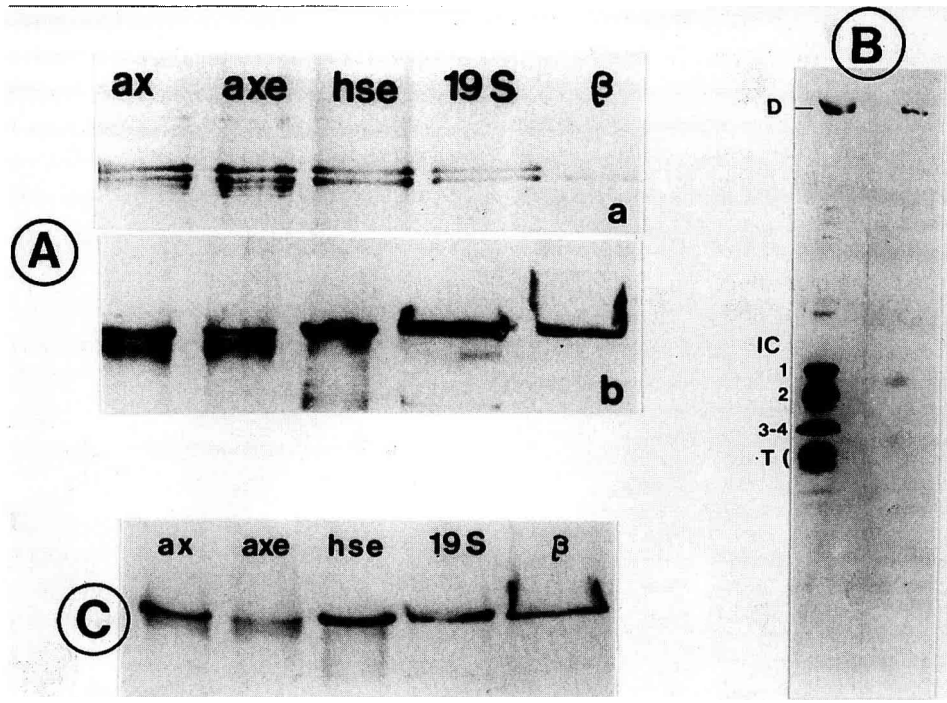

Fig 1. Trout sperm β dynein heavy chain antibody purification. Panel A (a and b): Intact isolated trout sperm axonemes (ax), high salt extracted axonemes (axe), high salt extract (hse), 19S sucrose gradient purified dynein (19S) and its purified β subunit (β) were analysed on a 4% polyacrylamide gel containing 2M urea to separate the heavy chains (silver-stained gel, in a). The equivalent nitrocellulose replica (in b) was probed with the anti-β whole polyclonal. The β heavy chain clearly reacted while the α chain did not. Panel B: Nitrocellulose replica of a 6–16% SDS-PAGE gel of intact isolated axonemes. The left lane shows that the whole polyclonal reacted also with the intermediate chains (IC1, 2, 3–4) and the tubulins (T). The right lane shows that after affinity purification only the dynein heavy chain (D) reacted. Panel C: The affinity-purified antibody was tested on the replica of a 4% gel to show the high specificity of the reaction obtained with the β heavy chain.

The polyclonal antibody obtained with the whole β subunit was tested for its reactivity against trout sperm isolated axonemes, high salt extracted axonemes, high salt extract, purified 19S dynein and the purified β subunit (fig 1, panel A, a). On a nitrocellulose replica of a 4% SDS-PAGE, the polyclonal antibody recognised strongly only one of the heavy chains, which appeared to be the β heavy chain (fig 1, panel A, b).

On a 6–15% gel replica of the intact axonemes the intermediate chains and the α and β tubulins also reacted with this serum (fig 1, panel B, left lane), showing that they contaminated the purified β subunit fractions of the low ionic strength sucrose gradient.

The polyclonal antibody was used to obtain a specific anti-β heavy chain antibody by blot-affinity purification (Olmsted, 1981). The specificity of the affinity-purified antibody was tested: this antibody recognised exclusively the β heavy chain among the axonemal heavy chains of the trout sperm flagella (fig 1, panel C) and no further cross-reactivity was observed with the intermediate chains or the tubulins on the

6–16% replica of intact axonemes (fig 1, panel B right lane).

Immunological cross-reaction with flagellar outer arm dynein heavy chains

With the blot-affinity-purified anti-trout β heavy chain antibody, we probed the sea urchin 21S outer arm dynein and its isolated β heavy chain (fig 2, panel A, left). A strong cross-reaction with only one of the sea urchin dynein heavy chains was observed (fig 2, panel A, right). Since it was the lower of the heavy chains that reacted, and also because the reaction was obtained with the enriched β heavy chain fraction, this strongly suggested that it was this chain, and not the α heavy chain, that cross-reacted.

Ram sperm possesses two major high molecular weight peptides at about 460 kDa and 430 kDa, which migrated close to the trout α and β heavy chains (fig 2, panel B, left) and remained tightly associated with the cell cytoskeleton after demembranation

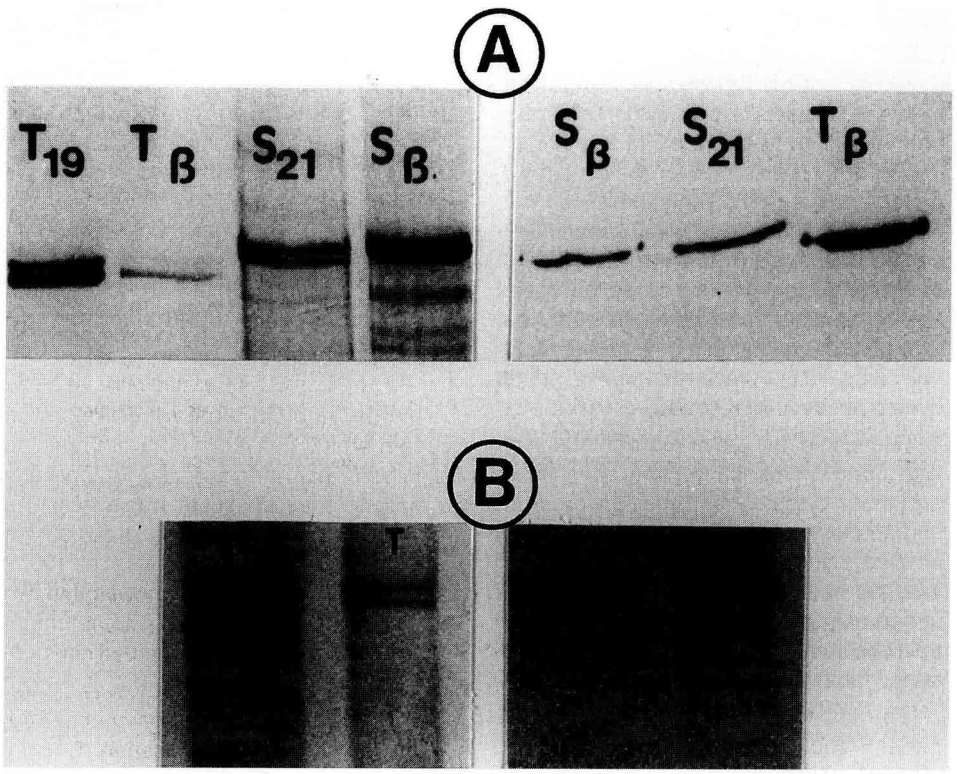

Fig 2. Immunological cross-reaction with sea urchin and ram sperm dynein heavy chain. Panel A: Sea urchin 21S sperm dynein (S_{21}) and its purified β/IC subunit (Sβ) were run on a 4% SDS polyacrylamide gel (left). Trout sperm 19S (T_{19}) and its β subunit ($T_β$) were used as molecular weight markers and also as a positive control on the Western blot (right). When the blot was probed with the affinity purified anti-trout β heavy chain antibody, the sea urchin sperm β heavy chain clearly reacted. Panel B: left: 4% polyacrylamide gel, 2M urea of ram sperm (R). Among the heavy chains only the one situated at about the same position as the trout β heavy chain (Tβ) cross-reacted (right). 1–5 x 10^6 spermatozoa/lane were loaded.

by Triton X 100 (not shown). The affinity purified anti-β dynein heavy chain antibody was used to probe an equivalent nitrocellulose replica (fig 2, panel B, right). The antibody reacted specifically with the lower of the high molecular weight polypeptides. This heavy chain was at about 430 kDa and migrated at almost the same position as the trout β heavy chain.

Absence of immunological cross-reaction with cytoplasmic dynein heavy chains

When the trout anti-β heavy chain affinity purified antibody was used to probe the high molecular weight region of a 4% gel of the low speed extracts and the cytosolic extracts obtained from trout brain (fig 3, panel A, upper part), no reaction was observed (fig 3, panel A, lower part). The same result was obtained when the total anti-β subunit antiserum was used (not shown). The high molecular weight proteins of the low speed and cytosolic extracts obtained from trout liver (in order to obtain more starting materials) also did not react with the trout sperm dynein antibody (not shown).

Because of the cross-reaction observed with a ram sperm heavy chain, sheep brain and testis cytoplasmic dyneins were purified and tested with the anti-β heavy chain antibody. No cross-reaction was observed with the heavy chains present in the low speed extract, the cytosolic extract or the fractions from the sucrose gradient that contained the cytoplasmic dynein.

The upper and lower parts of figure 3 panel B show respectively the 4% silver-stained gel and the nitrocellulose replica obtained with the brain cytoplasmic dynein. A similar result was obtained with the low speed extract and cytoplasmic dynein from mammalian testis (not shown). No reaction was observed with the same preparations when the whole anti-β subunit antibody was used. The absence of cross-reaction was not due to a lack of cytoplasmic dynein in the extract since a monoclonal directed against the cytoplasmic dynein heavy chain (Steuer et al, 1990) reacted with the heavy chain present in the sucrose gradient (not shown).

DISCUSSION

Our results showed that the α and β heavy chains within the same trout sperm outer arm dynein were immunologically distinct isoforms. They also showed that the epitope(s) recognized was shared by the β heavy chain from sea urchin sperm flagella and one heavy chain of ram sperm. A cross-reaction was also observed with one heavy chain of demembranated boar sperm which was demonstrated to be a dynein heavy chain (Gatti and Dacheux, 1994, and unpublished observations). These results agreed with and emphasized those previously obtained by Ogawa et al (1990). These authors showed that the sea urchin 21S outer arm dynein was also composed of two different isoforms using an affinity-purified antibody against the β heavy chain of sea urchin sperm flagella.

Immunological similarities between dynein heavy chains of different species have been reported previously. An antibody against the sea urchin outer arm dynein was found to be reactive against trout sperm axonemal heavy chains (Ogawa et al, 1980). Yoshida et al (1989) also observed that two high molecular weight polypeptides from rat sperm cross-reacted with a blot-affinity-purified antibody against a sea urchin sperm dynein heavy chain.

Our affinity antibody recognised heavy chains of the flagellar dynein from different species but did not cross-react with the cytoplasmic dynein from trout and sheep. This lack of cross-reaction between cytoplasmic and outer arm dynein heavy chains shows that divergence exists between these two families of heavy chains, certainly due to

the fact that the first duplication of the dynein heavy chains, gene gave rise to the axonemal and the cytoplasmic dynein (Tanaka et al, 1995). Asai et al (1994) have demonstrated that a specific motif contained within the dynein heavy chain sequence (named motif A), located near the P loop forming the ATP catalytic site, was conserved between axonemal dyneins from *Paramecium tetraurelia* (DHC-6), *Chlamydomonas* (α, β, γ heavy chains), and also the β heavy chain of sea urchin (see also Wilkerson et al, 1994). This motif was not found on the cytoplasmic dynein heavy chains. These authors also reported that the antibody directed against the corresponding peptide sequence recognised axonemal dynein heavy chain from sea urchin and bull sperm but not from cytoplasmic dynein.

These data suggest that the affinity-purified trout β heavy chain antibody recognised this type of epitope, maintained throughout evolution only on certain isoforms of the axonemal outer arm dynein heavy chain.

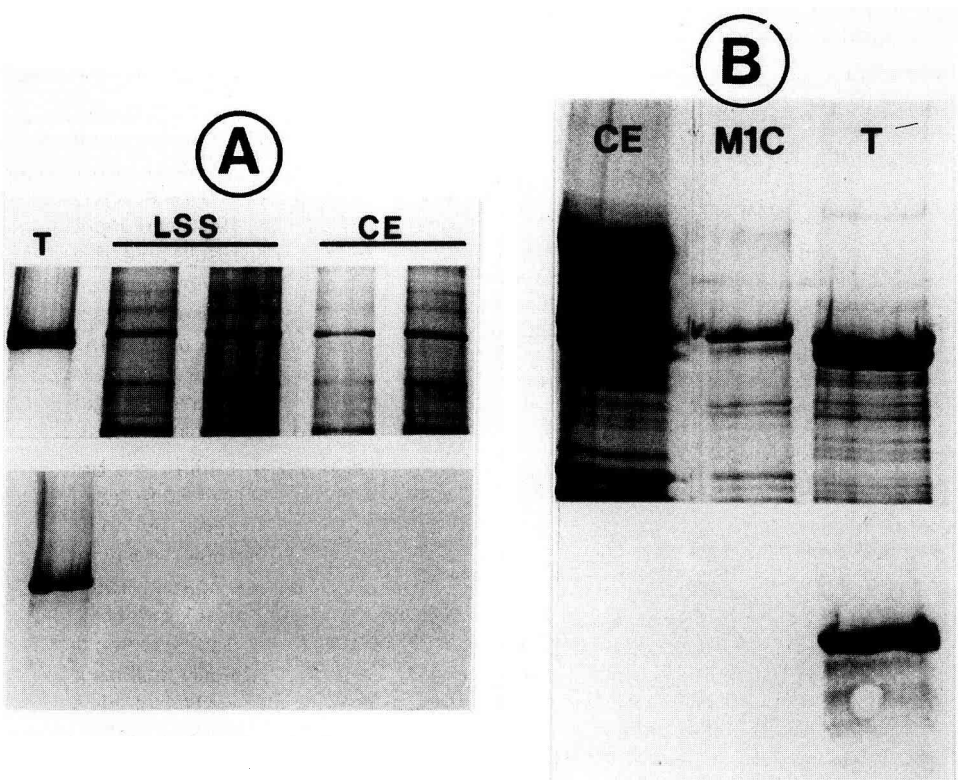

Fig 3. Lack of immunological cross-reaction with the cytoplasmic dynein. Panel A: Low speed supernatant (LSS) and the cytosolic extract (CE) from five trout brains were analysed on a 4% gel containing 2M urea (upper part). Trout sperm 19 S dynein (T) was used as molecular weight standard and as positive control on the blot (lower part) tested with the affinity-purified anti-β heavy chain antibody. No cross-reaction was observed with the high molecular weight proteins of the brain extracts. The second lanes of LSS and CE were loaded with twice as much proteins as the first lanes. Panel B: Sheep brain cellular extract (CE) and the peak fraction of brain cytoplasmic dynein purified by sucrose gradient centrifugation (M1C) were run on a 4% gel containing 2 M urea (upper part). No cross-reactivity was observed on the Western blot probed with the anti-trout β heavy chain (lower part). (T) Trout sperm 19S dynein.

This high level of conservation indicates that perhaps this sequence plays a special role in the flagellar beating process, or is necessary to maintain the structure of the outer arm within the axoneme. Indeed, a functional difference exists between the heavy chains derived from one outer arm dynein (Moss et al, 1992a, b) and also in the roles played by the α, β and γ heavy chains in the outer arm assembly in *Chlamydomonas* (Witman et al, 1994).

A further study to obtain the sequence of the epitope recognised by this affinity-purified antibody on the heavy chain of several different species will be necessary in order to analyse its specific function in either the beating process or the outer arm dynein assembly.

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