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Is Outer Arm Dynein Intermediate Chain 1 Multifunctional?

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The outer arm dynein of sea urchin sperm axoneme contains three intermediate chains (IC1, IC2, and IC3; M_r 128,000, 98,000, and 74,000, respectively). IC2 and IC3 are members of the WD family; the WD motif is responsible for a protein-protein interaction. We describe here the molecular cloning of IC1. IC1 has a unique primary structure, the N-terminal part is homologous to the sequence of thioredoxin, the middle part consists of three repetitive sequences homologous to the sequence of nucleoside diphosphate kinase, and the C-terminal part contains a high proportion of negatively charged glutamic acid residues. Thus, IC1 is a novel dynein intermediate chain distinct from IC2 and IC3 and may be a multifunctional protein. The thioredoxin-related part of IC1 is more closely related to those of two redox-active *Chlamydomonas* light chains than thioredoxin. Antibodies were prepared against the N-terminal and middle domains of IC1 expressed as His-tagged proteins in bacteria. These antibodies cross-reacted with some dynein polypeptides (potential homologues of IC1) from distantly related species. We propose here that the three intermediate chains are the basic core units of sperm outer arm dynein because of their ubiquitous existence. The recombinant thioredoxin-related part of IC1 and outer arm dyneins from sea urchin and distantly related species were specifically bound to and eluted from a phenylarsine oxide affinity column with 2-mercaptoethanol, indicating that they contain vicinal dithiols competent to undergo reversible oxidation/reduction.

INTRODUCTION

Dyneins are motor proteins responsible for various microtubule-based cell motilities and can be categorized into two subfamilies: axonemal and cytoplasmic dyneins. In fact, dynein is a huge supramolecular complex consisting of three polypeptide groups classified according to size: dynein heavy, intermediate, and light chains (abbreviated as DHCs[1], ICs, and LCs, respectively; for a review, Witman *et al.*, 1994). DHCs are very large polypeptides (M_r 520,000) and contain the motor and microtubule-binding domains of dynein (for a review, Vallee, 1993). The deduced amino acid sequences of all DHCs cloned to date contain four P-loop sequences for ATP binding in the middle region of the polypeptide. The N-terminal 1400 amino acid residues are thought to be the cargo-binding region and are different between axonemal and cytoplasmic subfamilies. The C-terminal two-thirds is thought to be the motor domain of dyneins (for a review, Ogawa, 1992), and this region is conserved among DHCs.

As for the ICs, those of axonemal outer arm dynein are likely to be homologous to those of cytoplasmic dynein (Paschal *et al.*, 1993). *Chlamydomonas* outer arm dynein has two kinds of ICs, referred to as IC78 and IC69 (this chain is also referred to as IC70 [Mitchell and Kang, 1991]). They have been shown to be located at the base of the solubilized dynein molecule (King and Witman, 1990). IC78 plays a role in the structural association of dynein with the doublet microtubules

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(King *et al.*, 1991, 1995), whereas IC69, which was the first IC to be cloned, may be required for the regulation of dynein activity (Mitchell and Kang, 1991, 1993). Rat brain cytoplasmic dynein IC is referred to as IC74, exists as three different variants, which may be due to alternative splicing (Paschal *et al.*, 1993). Interestingly, the C-terminal sequence of *Chlamydomonas* IC69 is homologous to cytoplasmic IC74 (Paschal *et al.*, 1993). Unlike *Chlamydomonas*, sea urchin sperm outer arm dynein has three ICs: IC1, IC2, and IC3. Ogawa *et al.* (1995) isolated two cDNA clones encoding IC2 and IC3 that were shown to have similar epitopes to *Chlamydomonas* IC78 and IC69, respectively. The most important feature of these ICs is that they are members of the WD family; WD sequences are responsible for certain protein-protein interactions (Neer *et al.*, 1994; Ogawa *et al.*, 1995; Wilkerson *et al.*, 1995).

As mentioned above, although *Chlamydomonas* dynein has two ICs that are members of the WD family, three ICs are present in dyneins from animals such as sea urchin (Tang *et al.*, 1982), oyster (Wada *et al.*, 1992), and clam *Spisula solidissima* (Stephens and Prior, 1992, 1995). The difference is the presence of an additional IC, referred to as IC1, whose M_r is about 130,000 in the case of these animals. Sea urchin dynein IC1 is tightly associated with β-DHC, and the β-DHC/IC1 complex translocates microtubules in vitro, functioning as a force-generating unit (Sale and Fox, 1988). It is suggested that the changes in interaction between β-DHC and IC1 are involved in the ATP hydrolysis cycle by the complex (Inaba, 1994 and 1995). Based on immunological analysis, Gagnon *et al.* (1994) suggested that IC1 might play a dynamic and crucial role in flagellar bending and/or wave propagation. If so, homologues of IC1 should be ubiquitously present in flagella of any species, including *Chlamydomonas*. However, in the case of animals such as rainbow trout (Gatti *et al.*, 1989; King *et al.*, 1990) and the mussel *Mytilus* (Stephens and Prior, 1992), IC1-like polypeptides were either missing or substoichiometirc to other ICs. Thus, it is an important question why the occurrence of IC1 does not appear to be ubiquitous.

To shed light on its role in dynein structure and function, we determined the sequence of sea urchin outer arm dynein IC1. The deduced amino acid sequence of IC1 has a unique feature in that the N-terminal region is homologous to the sequence of thioredoxin, the midregion consists of three repetitive sequences homologous to the sequence of nucleoside diphosphate (NDP) kinase, and that the C-terminal region contains a high proportion of negatively charged glutamic acid residues. Thus, IC1 is a novel family of ICs distinct from IC2 and IC3 and may be multifunctional. The N-terminal and middle regions were separately expressed in bacteria for raising antibodies. These antibodies cross-reacted with some dynein polypeptides (potential homologues of IC1) from distantly related species. Based on the present results, we propose that the outer arm dynein of animal sperm has three types of ICs.

MATERIALS AND METHODS

Sea Urchin Testis cDNA Library

Since monoclonal antibody (D52) against sea urchin sperm flagellar dynein IC1 did not react with any sea urchin embryonic ciliary proteins (Ogawa *et al.*, 1990), a testis cDNA library was substituted for the unfertilized egg cDNA library that had been used previously for molecular cloning of ciliary β-DHC (Ogawa, 1991), IC2, and IC3 (Ogawa *et al.*, 1995). Total RNA was extracted from a testis of one male sea urchin *Anthocidaris crassispina* using the guanidium isothiocyanate method. cDNA synthesis was performed by priming poly(A)$^+$ RNA with oligo(dT) using the Time-Saver cDNA Synthesis kit (Pharmacia, Uppsala, Sweden). Double-stranded cDNAs were ligated into the *Eco*RI site of λgt11. This library contained 5×10^6 independent clones per ml. Phage DNA isolation and the subcloning of cDNA inserts into plasmids were performed according to basic procedures (Sambrook *et al.*, 1989).

Sequencing

The cDNA inserts in the λ vector were subcloned into pBluescript IIKS+ (Stratagene, La Jolla, CA) at the *Not*I site. Plasmids with the cDNA in opposite orientations were obtained and a number of nested deletions were generated from the DNAs using the *double-stranded* Nested Deletion kit (Pharmacia). DNA sequences were determined with Sanger's chain-termination method using Sequenase Ver. 2 (United States Biochemical, Cleveland, OH) and double-stranded DNA as template.

Northern Blot

For Northern blots, poly(A)$^+$ RNA (3 μg/lane) was fractionated on formaldehyde-1% agarose gel (Sambrook *et al.*, 1988) and transferred to Hybond N+ filters (Amersham, Buckinghamshire, England) in 20× SSC. After UV cross-linking, hybridization was carried out at 65°C in a rapid hybridization buffer (Amersham) for 6 h. The filter was then washed with decreasing concentrations of SSC: 2× SSC-0.1% SDS for 30 min followed by 0.1× SSC-0.1% SDS overnight at 65°C.

Preparation of Sperm Axonemes

Semen of mature rainbow trout, *Oncorhynchus mykiss*, was obtained according to the method described previously (Inaba and Morisawa, 1991). Sperm axonemes were prepared as described previously (Inaba and Morisawa, 1992).

Sperm of the ascidian, *Halocynthia roretzi*, were obtained from an adult that was stimulated for spawning by light and temperature (Hoshi *et al.*, 1981). Testes were surgically removed and minced in filtered sea water on ice to release sperm. The sperm suspension was filtered through a nylon mesh (100 μm) to remove eggs and connective tissues and centrifuged at 9000 × *g* for 5 min. After the pellet was washed twice with filtered sea water, sperm were homogenized in filtered sea water and the homogenate was centrifuged at 3000 × *g* for 5 min to sediment detached sperm heads. The supernatant was centrifuged at 15,000 × *g* for 10 min, and the pellet of sperm flagella was suspended in a demembranation solution (150 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol (DTT), and 0.1% Triton X-100). The suspension was centrifuged at 15,000 × *g* for 10 min, and the pellet was washed twice with Triton-free demembranation solution. The resulting axonemes were used for the extraction of dynein.

Preparation of axonemes from the sperm of the mussel, *Mytilus edulis*, was performed according to the method described by Ste-

phens and Prior (1992). Testes were minced in filtered sea water on ice, stirred for 10 min at 4°C, and filtered through a nylon mesh (100 μm). The filtrate was centrifuged at 6000 × *g* for 10 min. The sperm pellet was homogenized in filtered sea water and centrifuged at 3000 × *g* for 5 min. The supernatant was recovered and further centrifuged at 15,000 × *g* for 10 min to collect sperm flagella. Axonemes were obtained in the same manner as described above.

Isolation of Dynein

The procedure for isolation of dyneins from the above-mentioned three species was essentially the same as the methods described for sea urchin sperm dynein (Inaba and Mohri, 1989). Axonemes were suspended in approximately five volumes of extraction buffer (Triton-free demembranation solution with higher KCl concentration, 0.6 M) and incubated on ice for 30 min. The suspension was centrifuged at 22,000 × *g* for 10 min, and the resulting supernatant was further centrifuged at 100,000 × *g* for 30 min. The clear supernatant was loaded onto a 26-ml 5–20% linear sucrose density gradient and centrifuged at 26,000 rpm for 14 h in a Hitachi RPS27 rotor. Fractions of 1.5 ml were collected from the bottom of the tube. Protein concentration and ATPase activity in each fraction were determined as described previously (Inaba and Mohri, 1989). The sedimentation coefficient was estimated from the relative mobilities in sucrose density gradient using thyroglobulin (19S), catalase (11.3S), and bovine serum albumin (4.7S) as markers.

Measurement of NDP Kinase Activity

ADP and GTP were purchased from Boehringer Mannheim (Mannheim, Germany) and were purified by DEAE-Sephadex column chromatography with triethylammonium-bicarbonate buffer gradient as described previously (Shimizu and Furusawa, 1986). The resultant triethylammonium salts of the nucleotides were converted into corresponding sodium salts by small Dowex AG-50W columns and pH titration with NaOH.

Ciliary 22 S dynein from *Tetrahymena thermophila* was prepared as described previously (Porter and Johnson, 1983). This dynein has little, if any, adenylate kinase (Holzbaur and Johnson, 1986) and NDP kinase (this study) activities and exhibits low GTPase activity (Shimizu, 1987).

The assay mixture contained 50 mM MOPS-NaOH (pH 7.0), 50 mM NaCl, 4 mM $MgCl_2$, 0.5 mM GTP, 0.1 mM ADP, 0.05 mg/ml *Tetrahymena* 22 S dynein, and an enzyme fraction. After incubation at 25°C for 10 min, an aliquot of the assay mixture was withdrawn and mixed with a final concentration of 0.3 M perchloric acid. Inorganic phosphate produced was determined with the modified malachite green method (Kodama *et al.*, 1986).

To estimate the NDP kinase activity, it was necessary to assess the adenylate kinase contamination in the enzyme fraction as well as the apparent GTPase activity. Thus, an assay without GTP (for adenylate kinase estimation) and another without ADP (for GTPase estimation) were also performed. These values were subtracted from the apparent activity of the complete system described above.

Phenylarsine Oxide (PAO) Affinity Chromatography

A PAO-conjugated agarose (ThioBond) was purchased from Invitrogen (San Diego, CA). Recombinant polypeptide and outer arm dyneins were subjected to this column according to the method of Patel-King *et al.* (1996). ThioBond resin was activated with 100 mM 2-mercaptoethanol and equilibrated with TENT buffer (Patel-King *et al.*, 1996). The samples were reduced with 1 mM DTT for 60 min at 4°C and dialyzed overnight against TENT buffer to remove DTT. After washing with TENT buffer, proteins containing active vicinal dithiols were eluted with 0.5 M 2-mercaptoethanol. Equivalent volumes of the initial, unbound, and eluted fractions were electrophoresed and stained with Coomassie brilliant blue or subjected to Western blotting. The relative intensities of DHC bands in each fraction were determined with a Densitograph (ATTO, Tokyo, Japan).

Antibodies

Affinity-purified antibodies directed against the three ICs of sea urchin sperm outer arm dynein were the same as those reported previously (Ogawa *et al.*, 1988). Anti-IC1 monoclonal antibody (D52) was also reported previously, reacted with sperm IC1 but did not react with any ciliary proteins (Ogawa *et al.*, 1990).

Detection of Immunocomplexes

For Western blots and plaque blots incubated with rabbit antibodies, alkaline phosphatase (AP)-conjugated anti-rabbit IgG antibodies (Cappel, West Chester, PA) were used as second antibodies at a 1:2000 dilution. For those incubated with mouse monoclonal antibody (D52), AP-conjugated anti-mouse IgM (Sigma, St. Louis, MO) was used at a 1:2000 dilution. Immunocomplexes were detected with 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Mierendorf *et al.*, 1987). For Western blots, prestained polypeptides of M_r 180,000, 116,000, 84,000, 58,000, 48,500, and 36,500 (Sigma) were used as markers.

Direct Amino Acid Sequencing of IC1

IC1 was prepared from the 21S outer arm dynein purified from sea urchin (*A. crassispina*) sperm axonemes by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was stained with Coomassie brilliant blue and then destained. IC1 appeared as two closely spaced bands. The band with the smaller M_r was excised and cleaved with 10 mg/ml cyanogen bromide (CNBr) in 70% formic acid for 16 h. The CNBr solution was removed and the strip was rinsed with 70% formic acid for 1 h. The CNBr and formic acid solutions were combined and dried under vacuum. The resultant peptides were separated by high-pressure liquid chromatography and sequenced in a sequencer (model Procise; Applied Biosystems, Foster City, OR).

Sequence Analysis

The GCG programs COMPARE and DOTPLOT were used to generate dot plot comparisons. The percentage of identity and similarity between two proteins was calculated using the GCG program GAP or BESTFIT. The secondary structure was analyzed with the GCG program PEPPLOT. Pairwise and multiple sequence alignments were performed using the CLUSTAL W program (Thompson *et al.*, 1994).

RESULTS

cDNA Sequence of Sea Urchin Outer Arm Dynein IC1

The cDNA sequence of IC1 was determined from five overlapping clones (Figure 1). The λgt11 cDNA library prepared from *A. crassispina* testis RNA was screened with affinity-purified antibodies against IC1 (Ogawa *et al.*, 1988). Five positive clones were isolated from 2 × 10^5 independent clones. The clones were further examined by the monoclonal antibody against sea urchin IC1 (D52; Ogawa *et al.*, 1990). Two clones were positive. They were subcloned into the *Not*I site of pBluescript IIKS+ to yield pJY121 and pJY122. pJY121 consisted of a sequence corresponding to bases 31–2079 of the final sequence. There was an in-frame deletion (bases 2017–2062) in this clone. The other

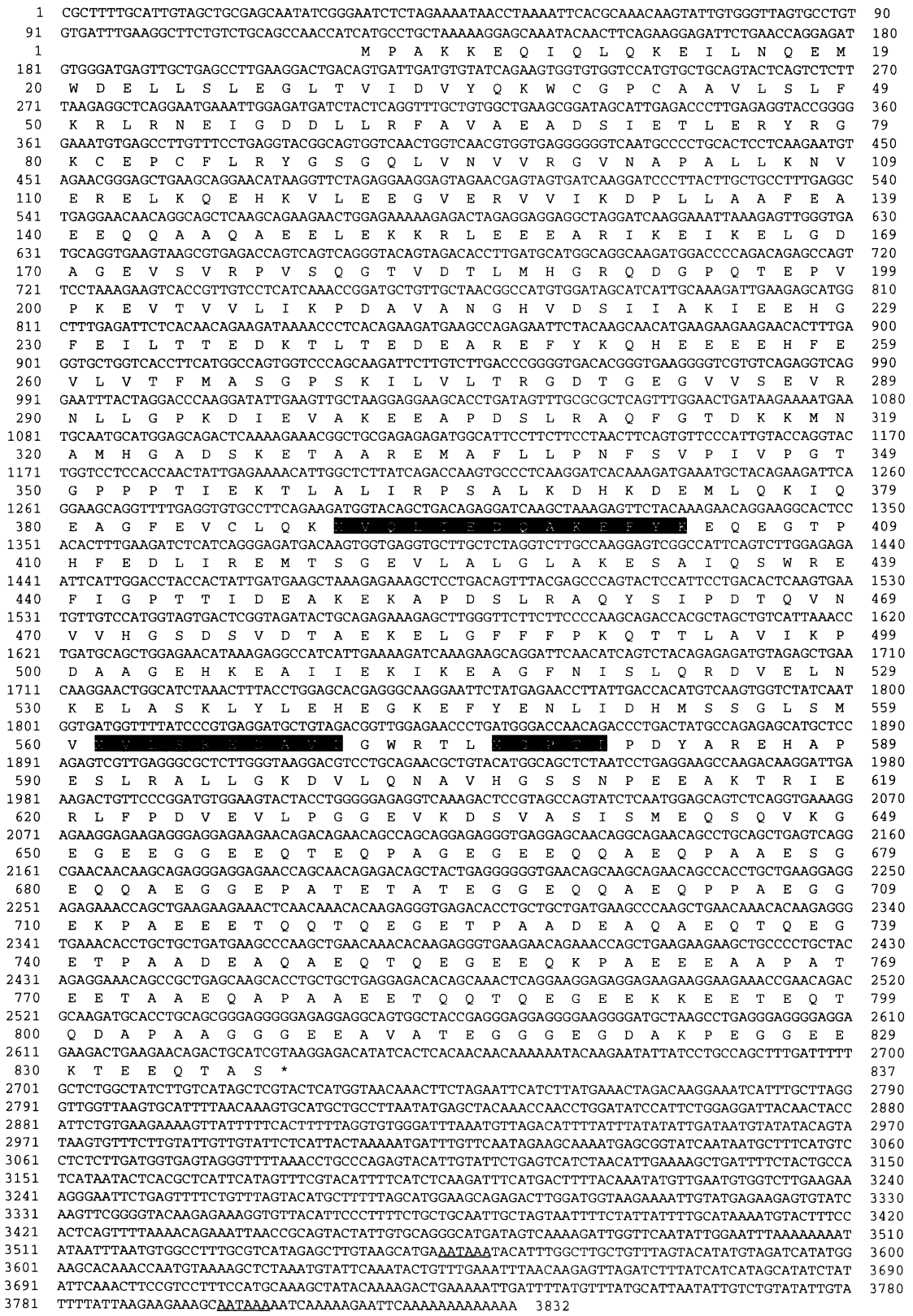

Figure 1.

clone, pJY122, consisted of a sequence corresponding to bases 1–2073.

A *Pst*I fragment of pJY121 (bases 1560–1925) was used as a probe for obtaining cDNA clones extending in the 3′ direction. Seven positive clones were obtained from 2×10^5 independent clones of the original cDNA library. They were subcloned to yield pAG281-287 and sequenced. pAG283 consisted of a sequence corresponding to bases 928-2599.

A *Pst*I fragment of pAG283 (bases 2145–2532) was used as a probe for obtaining cDNA clones extending in the 3′ direction. A number of positive clones were obtained from 2×10^5 independent clones of the original cDNA library. Fifteen clones were purified for subsequent analysis. The insert DNA of each clone was amplified by PCR between forward or reverse primer for λgt11 vector and sequence-specific sense primer (5′-AAG GAA GAA ACC GAA CAG ACG-3; bases 2501–2521) to assess the degree of extension of cDNA in the 3′ direction. Two candidate clones were subcloned to yield pJN71 and pOC1415 corresponding to bases 963-3826 and 2435–3832, respectively. Both clones had a poly(A)-segment at the 3′ terminus.

Assuming that translation initiation occurs at the first in-frame AUG triplet (position 125), the open reading frame encoded a polypeptide of 837 amino acid residues with a predicted isoelectric point of 4.24 and a predicted molecular mass of 91.6 kDa. The open reading frame terminated with a stop codon (UAA, indicated with an asterisk in Figure 1) and was followed by a 3′ untranslated sequence that includes two polyadenylation signals (AAUAAA, underlined in Figure 1). Because of the occurrence of an in-frame deletion of bases 2017–2062 in some clones (pJY121 and pAG283), there could be another version of IC1 lacking 15 amino acid residues. Also, two identical repetitive sequences (QTQEGETPAADEAQAEQTQ-EGE) were found at the amino acids 719–740 and 735–756.

The Northern blot analysis of testis poly(A)$^+$ RNA probed with the *Pst*I fragment (bases 2145–2532) shows that the probe hybridized to a 3.8-kb band (Figure 2). The size of this band agrees well with that of the cDNA constructed from five overlapping clones.

Three peptides derived from IC1 by CNBr cleavage were sequenced. The sequences (with one-letter code) were xxxxEDQAKEFYK (where x is an unidentified amino acid), xLSREDAVD, and xPTD. Since CNBr cleaves the C-side of methionine, they were supposed to be MxxxxEDQAKEFYK, MxLSREDAVD, and Mx-PTD, respectively. These sequences actually appeared at the amino acids 390–403, 561–570, and 576–580 (boxes, Figure 1), respectively. From these results, we conclude that this overlapped sequence encodes the sea urchin sperm outer arm dynein IC1.

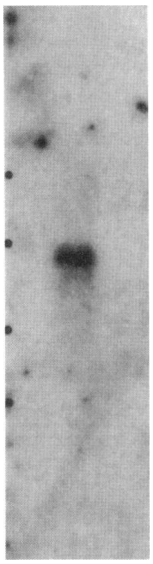

Figure 2. Northern blot analysis of sea urchin testis poly(A)$^+$ RNA. Dots are the positions of RNA markers (7.46, 4.40, 2.37, and 1.35 kb from the top, respectively; Life Technologies, Gaithersburg, MD) in the neighboring lane.

Thioredoxin-related Region of IC1

When we submitted this sequence of sea urchin outer arm dynein IC1 to the databases on August 24, 1995, a search of the databases using the BLAST program (Altschul *et al.*, 1990) was also performed to reveal that the N-terminal region of the deduced amino acid sequence of IC1 was closely related to the sequence of thioredoxin from a variety of organisms. The sequence comparison of IC1 and mouse thioredoxin (accession number X77585) performed with the program GAP using the default settings showed that they exhibited 28.2% identity and 43.7% similarity. Figure 3A shows a dot matrix sequence comparison of sea urchin IC1 versus mouse thioredoxin. Sequence homology was limited to the N-terminal region of IC1. Therefore, the corresponding region (amino acids 1–130) is referred to as the thioredoxin-related (TRXR) region in this article.

Recently, Patel-King *et al.* (1996) described the sequences of the two redox-active *Chlamydomonas* outer arm dynein LCs (LC14 and LC16). These two LCs are homologous to each other and the sequence comparison shows that they are 33.9% identical and 51.2% similar. Multiple sequence alignment of sea urchin IC1 and the two *Chlamydomonas* LCs were performed using the CLUSTAL W program (Figure 4). The thioredoxin consensus pattern is [S/T/A]-x-

Figure 1 (cont). Nucleotide and predicted amino acid sequences of the full-length cDNA that encodes the sea urchin sperm outer arm dynein IC1. The boxes indicate the amino acid sequences determined by direct peptide analysis. The sequence reported here will appear in the GSDB, DDBJ, EMBL, and NCBI Nucleotide Sequence Databases under the accession number D63884.

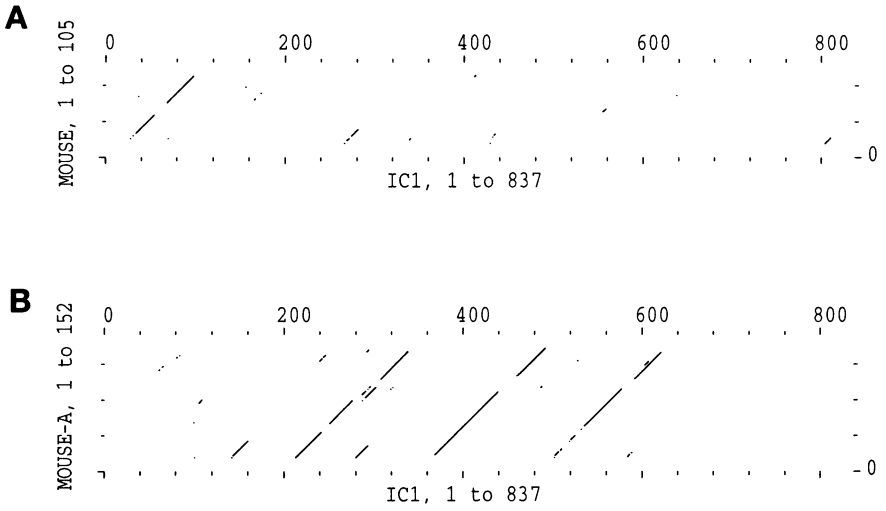

Figure 3. Dot plot comparisons of sea urchin IC1 versus mouse thioredoxin (A) and versus mouse NDP kinase A (B). The dot plots were generated by the GCG Program COMPARE using a window size of 30 and a stringency of 14.

[W/G]-C-[A/G/V]-[P/H]-C, where two cysteines are for the redox-active bond, according to the PROSITE databases (Bairoch, 1991). The thioredoxin pattern perfectly matched in the case of LC16, but position 1 [S/T/A] did not match in the cases of LC14 and IC1. Sequence comparison of IC1 and LC14 shows that they are 36.2% identical and 52.0% similar, whereas the N-terminus of IC1 and LC16 are 30.2% identical and 51.3% similar. From these analyses, the N-terminal region of sea urchin IC1 is more closely related to *Chlamydomonas* LC14 and LC16 than to any thioredoxin and more closely related to LC14 than to LC16.

NDP Kinase-related Region of IC1

A search of databases using the BLAST program also revealed that the middle region of IC1 is closely related to the sequence of NDP kinases. IC1 and mouse type A NDP kinase were compared using the BESTFIT program. The amino acid positions 353–515 of IC1 and the sequence of the mouse enzyme were 30.7% identical and 60.0% similar (Figure 5A). Surprisingly, a dot plot sequence comparison revealed that IC1 contained not one but three NDP kinase-related (NDPKR) sequences (Figure 3B). These repetitive units are referred to as NDPKR-1 (amino acids 199–351), NDPKR-2 (amino acids 353–515), and NDPKR-3 (amino acids 515–643) from the N-terminus in the present article. The amino acids were 29.5% identical and 50% similar between NDPKR-1 and the mouse enzyme and 24.4% identical and 47.2% similar between NDPKR-3 and the mouse enzyme. The three sequence segments were aligned using the CLUSTAL W program (Figure 5B). The consensus pattern for NDP kinase in the PROSITE database is N-x-x-H-G-S-D-[S/A]-[L/I/V/M/R], where x is any amino acid, perfectly matched in the case of NDPKR-2. However, the residues at positions 4 and 7 and those at positions 5 and 6 did not match in the case of NDPKR-1 and NDPKR-3, respectively. The putative active site residue histidine (position 2), believed to be phosphorylated by the transfer of a terminal phosphate group from nucleoside triphosphate (NTP), exists in all segments.

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LC14    -------MAFITEIANEAQWKTEVLETPGTLQVVEVFQSHCGPCKAVQSTFKKL   (1-47)
LC16    MAAG--LPPVQYSIDTDADLETRL--AGKGLKVVEIFSEHCGPCKSVLPTFRRI   (1-50)
TRXR    MPAKKEQIQLQKEILNQEMWDELL--SLEGLTVIDVYQKHCGPCAAVLSLFKRL   (1-52)
                  * .       .  .   * *....  ***** .*   *...

LC14    YFDLND-RPLKFYSVSSER---LSSLKEYVGKCKPIFLFFKDGKQVEKIEGVKA   (48-97)
LC16    RLDKDDENALLFLTVCAEKCNFLETAKEHRGKSEPLFLLYRNGQLKARIEGANT   (51-104)
TRXR    RNEIGDD-LLRFAVAEADS---IETLERYRGKCEPCFLRYGSGQLVNVVRGVNA   (53-102)
           . *   * *    ..    . .      **. * ** .  *.    . *  .

LC14    PQLNRIVTELSGK---------NPPPAAPAAAPAAPAAEAS-----------   (98-129)
LC16    PALNQQVLSLTPANADVDDLEENPMYLAKMERERIARGETVKDAKGAKKGKK   (105-156)
TRXR    PALLKNVERELKQ----------EHKVLEEGVERVVIK--------------   (103-130)
        * * . *
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Figure 4. Multiple sequence alignments of sea urchin IC1, *Chlamydomonas* LC14, and LC16 using the program CLUSTAL W. The boxes indicate the consensus amino acid sequences for thioredoxins. Dashes indicate gaps introduced to obtain an optimum alignment. Asterisks indicate the amino acids identical in all sequences, whereas the dots indicate conservative replacements.

A

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D63884  353 PTIEKTLALIRPSAL.KDHKDEMLQKIQEAGFEVCLQKMVQLTEDQAKEF 401
            :. |:|:  |:|.:: ::  :|::.::::: || :.  |::| .||  ||
P15532    2 ANSERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVGLKFLQASEDLLKEH 51

          402 YKEQEGTPHFEDLIREMTSGEVLALGLAKESAIQSWREFIGPTTIDEAKE 451
              |.: .: | |.:|:: | ||.|:|:.:.  ..:.. | ::|.|. .:.|
           52 YTDLKDRPFFTGLVKYMHSGPVVAMVWEGLNVVKTGRVMLGETNPADSK. 100

          452 KAPDSLRAQYSIPDTQVMVVEPVDTAEKELGFFFPKQTTLAVIKPDA 501
                |:.:|::::|. .. |::||||||..||||::::|         .|:.
          101 ..PGTIRGDFCIQVGR.NIIHGSDKSAEKEISLWF.........QPEE 138

          502 AGEHKEAIIEKIKE 515
              .|.|..  :.| |
          139 LVEYKSCAQNWIYE 152
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B

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NDPKR-2  IEKTLALIRPSA-LKDHKDEMLQKIQEAGFEVCLQKMVQLTEDQAKE  (356-400)
NDPKR-3  KQTTLAVIKPDA-AGEHKEAIIEKIKEAGFNISLQRDVELNKELASK  (490-535)
NDPKR-1  KEVTVVLIKPDAVANGHVDSIIAKIEEHGFEILTTEDKTLTEDEARE  (201-247)
          . *. .*.* *    * . .. ** * ** .       *. . *

         FYKEQEGTPHFEDLIREMTSGEVLALGLAK----ESAIQSWREFIGP   (401-443)
         LYLEHEGKEFYENLIDHMSSGLSMVMVLSR----EDAVDGWRTLMGP   (536-578)
         FYKQHEEEEHFEVLVTFMASGPSKILVLTRGDTGEGVVSEVRNLLGP   (248-294)
          * ..*    .* *.  *.**    . *..    *  .   *  .**

         TTIDEAKEKAPDSLRAQYSIPDTQVMVVEPVDTAEKELGFFFP   (444-489)
         TDPDYAREHAPESLRALLG-KDVLQNAVHGSNPEEAKTRIERLFP   (579-623)
         KDIEVAKEEAPDSLRAQFG-TDKKMNAMHASDSKETAAREMAFLLP   (295-339)
            . *.* **.****     *   * .**.  . *   .    *
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Figure 5. (A) Comparisons of the NDPKR-2 of sea urchin IC1 (accession number D63884) and the entire sequence of mouse NDP kinase A (accession number P15532) using the GCG Program BESTFIT with the default settings. The boxes indicate the consensus amino acid sequences for NDP kinases. (B) Three-way comparison of the NDPKR sequences aligned using the CLUSTAL W program. Dashes indicate gaps introduced to obtain optimum alignment. The boxes indicate the consensus amino acid sequences for NDP kinase. Asterisks indicate amino acids identical in all sequences, whereas dots indicate conservative replacements. NDPKR-1 corresponds to the amino acids 199–351, NDPKR-2 to the amino acids 353–515, and NDPKR-3 to the amino acids 515–643.

The C-Terminal Region of IC1

The secondary structure of IC1 was visualized using the GCG program PEPPLOT (Figure 6). The top panel shows the secondary structure propensities defined by Chou and Fasman (1978). The solid line represents α helical propensity and the dotted line is for β strands. The helical hydrophobic moment was calculated as described by Eisenberg *et al.* (1984). In the middle panel, the solid and the dotted line correspond to the α and the β hydrophobic moments, respectively. The lower graph shows the Kyte and Doolittle hydropathy measure (1982). These features suggest that the C-terminal region of IC1 is, for the most part, α helical in nature and may be protruded from the protein.

There were no significant sequences homologous to the C-terminal region, but some similar sequences were found, e.g., the immediate-early protein of *Herpesvirus saimiri* (accession number Q01042), the cytochrome C1 precursor of *Paracoccus denitrificans* (P13627), the myristoylated alanine-rich kinase C substrate of chicken (P16527), and the submandibular gland secretary Glx-rich protein of rat (P08462). These sequences are highly abundant in glutamic acids, glutamines, or alanines. The resemblance of the C-terminal region of IC1 to those proteins may be solely due to a high proportion of glutamic acids.

Anti-TRXR Region and Anti-NDPKR Region Antibodies

To search for any homologues of IC1 in outer arm dyneins of distantly related species, we exploited the TRXR region (amino acids 1–130) and the NDPKR region (amino acids 131–615) to raise polyclonal antibodies. Lanes 2 and 3 of Figure 7 indicate successful production of anti-TRXR region and anti-NDPKR region antibodies, respectively. Lane 4 is a positive control of IC1 visualized using the monoclonal antibody (D52) against IC1.

As for the epitopes for anti-TRXR region antibodies, it was investigated whether they react with bacterial thioredoxin (Fermentas MBI) and recombinant *Chlamydomonas* LC14 and LC16 that were kindly provided by Dr. Stephen King. Blotted thioredoxin, LC14, and LC16 were not visualized using the antibodies. Thus, the thioredoxin consensus sequence represented by the sequence WCGPC would not be the epitopes

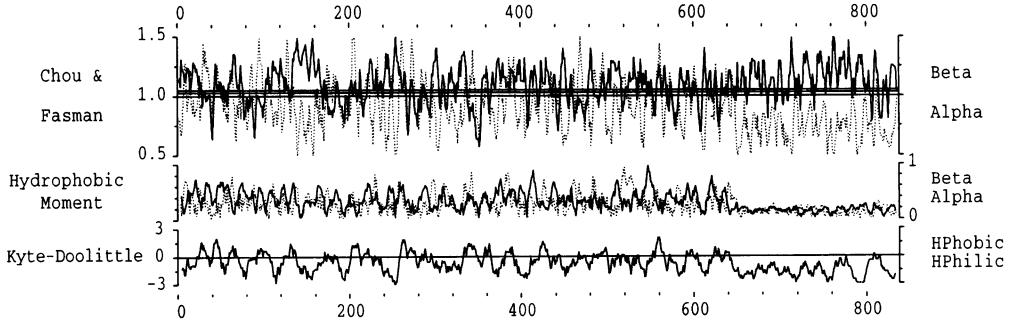

Figure 6. Structural features of IC1. The graphic representation was generated with the GCG program PEPPLOT on a Sun workstation.

for anti-TRXR region antibodies. Anti-thioredoxin monoclonal antibody (Invitrogen, San Diego, CA) also did not react with recombinant TRXR region either.

Sea urchin sperm outer arm dynein also contains LCs. In this species (*A. crassispina*), six bands of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa were identified by the silver staining of 16.5% SDS-polyacrylamide gel. Total numbers of outer arm dynein LCs coincided with those described in another species of sea urchin by Moss *et al.* (1992). To determine whether some of them contain the TRXR sequence of IC1, they were subjected to Western blotting. However, none of them reacted with anti-TRXR region antibodies. Thus, we conclude that none of the sea urchin outer arm dynein LCs contained the sequence shared with the TRXR region of IC1.

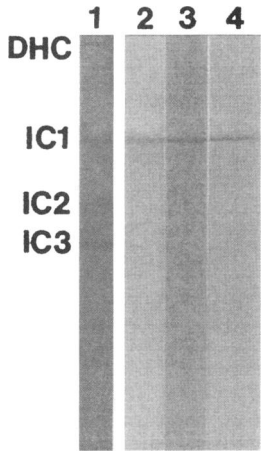

Figure 7. Evaluation of anti-NDPKR region and anti-TRXR region antibodies. Anti-NDPKR region antibodies were prepared as follows: pJY121 was digested with *Bam*HI so that the *Bam*HI fragment contained bases from position 515 (Figure 1) to the *Bam*HI site of vector. The corresponding amino acid residues are positions 131–615. This fragment was inserted into the *Bam*HI site of a His-tagged expression vector pQE31 (QIAGEN, Chatsworth, CA). His-tagged protein expression was induced by the addition of IPTG according to the manufacturer's protocol. The soluble fraction was partially purified on a Ni-NTA resin (QIAGEN). Recombinant protein of interest was electroeluted by LKB2014 Extraphor Electrophoretic Concentrator (LKB-Producter AB, Bromma, Sweden) after SDS-PAGE and used as the antigen. Anti-TRXR region antibodies were prepared as follows: the specific sense primer TCG *AGC TC*T AAG GAG CAA ATA CAA CT (*italics* show *Sac*I site introduced) and antisense primer CCG *AAG CTT* ATC CTT GAT CAC TAC TC (*italics* show *Hin*dIII site) were synthesized for amplifying DNA (bases 137–517) encoding the TRXR region. Thirty cycles of PCR were carried out on a 50-μl scale with a GeneAmp PCR System 9600 (Perkin Elmer-Cetus) in the 1× AmpliTaq buffer containing 100 μM dNTPs, 2 U AmpliTaq DNA polymerase (Perkin Elmer-Cetus), 0.5 μM each upstream and downstream primers, and template DNA (pJY121). The initial denaturation was performed at 94°C for 60 s. Denaturation during cycling was performed at 92°C for 60 s, primer annealing at 55°C for 90 s, and extension at 72°C for 120 s. Postextension was at 72°C for 7 min. The PCR product was electrophoresed in agarose gel, and the band of interest was purified by a SpinBind DNA Recovery System (FMC, Rockland, ME). The *Sac*I/*Hin*dIII fragment was inserted into a His-tagged expression vector pET29b+ (Novagen, Madison, WI) at the *Sac*I/*Hin*dIII site. A His-tagged protein expression was induced by the addition of IPTG according to the manufacturer's protocol. Recombinant protein was solubilized with 6 M urea and purified by a Ni-NTA resin. The eluate was used as the antigen. The 21S dynein was run in a 10% SDS-polyacrylamide gel whose stacking gel was made without a comb and transferred to Immobilon-P membrane. Lanes were cut out and visualized with anti-NDPKR region antibodies at a 1:1000 dilution (lane 2), anti-TRXR region antibodies at a 1:200 dilution (lane 3), and monoclonal antibody (D52) against IC1 (lane 4). Lane 1 is 21S dynein in a separate run whose blot was stained with Coomassie brilliant blue.

Occurrence of the TRXR Region and/or NDPKR Region in the ICs of Other Animals

Rainbow Trout *Oncorhynchus*. Gatti *et al.* (1988) and King *et al.* (1990) reported that the outer arm dynein of rainbow trout sperm has five ICs, which we have confirmed. Lane 6 of Figure 8A shows that the trout ICs are 89-, 79-, 65-, and 55-kDa polypeptides. The 65-kDa band is actually composed of two closely spaced bands. To compare the immunological similarities of these ICs to those of sea urchin sperm outer arm dynein, blots were processed with anti-NDPKR region (Figure 8A, lane 2), anti-IC2 (Figure 8A, lane 3), anti-IC3 (Figure 8A, lane 4), and anti-TRXR region (Figure 8A, lane 5) antibodies. The anti-NDPKR region antibodies reacted with the closely spaced 65-kDa bands (Figure 8A, indicated with the two dots along lanes 2 and 6) that have been previously referred to as trout IC3 and IC4 (Gatti *et al.*, 1988; King *et al.*, 1990). On the other hand, the anti-TRXR region antibodies did not react with either 65-kDa band. Thus, the present work demonstrates that an immunological homologue(s) of sea urchin IC1 truly exists in trout dynein. Also, we found that the anti-sea urchin IC2 and IC3 antibodies reacted with the 89- and 79-kDa bands that had previously been referred to as trout IC1 and IC2, respectively. The 55-kDa band reacted with a monoclonal anti-β-tubulin antibody (Amersham). Although Gatti *et al.* (1989) demonstrated that the trout IC5 band was electrophoretically distinct from tubulin and that it was not recognized by anti-tubulin monoclonal antibodies, it is possible that this band consists of an unidentified polypeptide plus tubulin that were indistinguishable under our electrophoretic condition. As described below, most of the 55-kDa protein was found not to associate with outer arm dynein (Figure 10, see lane e of the trout sample in the top panel). From these results, the trout polypeptide previously referred to as IC1 was proved to correspond to sea urchin IC2 and the trout IC2 corresponds to sea urchin IC3.

Ascidian *Halocynthia*. *Halocynthia* outer arm dynein contained 97-, 87-, and 55-kDa polypeptides as shown in lane 6 of Figure 8B, although they did not exist in a stoichiometric manner. The 55-kDa band cross-reacted with anti-β-tubulin antibody. As described below, most of the 55-kDa protein was found not to associate with outer arm dynein (Figure 10, see lane e of the *Halocynthia* sample of the bottom panel). The 87-kDa band could be separated into two bands under some conditions. Anti-NDPKR region and anti-TRXR region antibodies reacted with one major band indicated with the dot (Figure 8B, lane 2) that coincided with the 97-kDa band visualized with silver staining (indicated

with the dot along Figure 8B, lane 6). Several poorly defined bands also reacted with anti-NDPKR region antibodies. The anti-TRXR region antibodies bound only to the 97-kDa band (Figure 8B, lane 5). The anti-IC2 antibodies reacted with an 87-kDa band (Figure 8B, lane 3) and the anti-IC3 antibody also reacted with an 87-kDa band (Figure 8B, lane 4). However, the band visualized using anti-IC3 antibodies appeared to migrate slightly faster in the gel than the band visualized with anti-IC2 antibodies. Therefore, the 87-kDa band was likely to consist of the two polypeptides, *Halocynthia* IC2 and IC3, as described above. The present work demonstrates that *Halocynthia* outer arm dynein consists of three ICs homologous, or at least immunologically similar, to the three sea urchin ICs.

Mussel *Mytilus*. Lane 6 of Figure 8C shows the polypeptide components of the *Mytilus* sperm flagellar outer arm dynein. The 96- and 85-kDa bands were prominent and copurified with DHCs. They are apparently stoichiometric to each other and are likely to correspond to *Mytilus* IC2 and IC3 as described by Stephens and Prior (1992). *Mytilus* IC2 (96-kDa band) and IC3 (85-kDa band), in fact, reacted with antibodies against sea urchin IC2 and IC3, respectively, as shown in lanes 3 and 4. Several bands with M_r 122,000, 114,000, 106,000, 76,000, and 65,000 (indicated with the dots) were also enriched in the 20S dynein fraction. Anti-NDPKR region antibodies reacted with all of these bands (Figure 8C, lane 2). Thus, they might correspond to *Mytilus* IC1. The present work supported the observation of Stephens and Prior (1992) that the *Mytilus* outer arm dynein was likely to have a homologue(s) of IC1 in addition to IC2 and IC3. On the other hand, anti-TRXR region antibodies did not stain any bands (Figure 8C, lane 5).

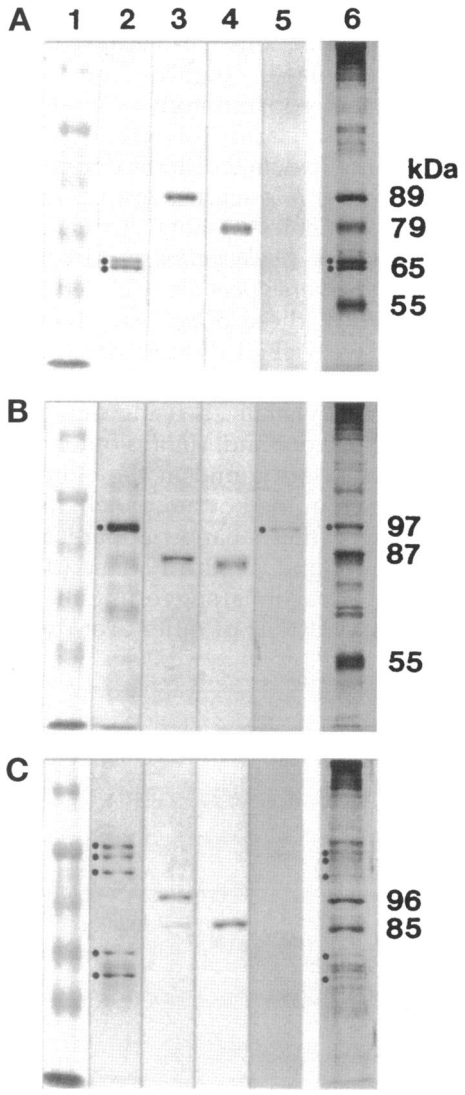

Figure 8. Visualization of three ICs contained in outer arm dyneins from rainbow trout (A), the ascidian *Halocynthia* (B), and the mussel *Mytilus* (C). Dyneins were electrophoresed in 10% SDS-polyacrylamide gel and transferred to NC filters. Each lane was cut out and visualized using the anti-NDPKR region (lane 2), anti-IC2 (lane 3), anti-IC3 (lane 4), and anti-TRXR region (lane 5) antibodies. In a separate run, proteins were visualized using silver staining (lane 6, A–C). Lane 1 shows protein markers (Sigma).

IC1 Contains Redox-sensitive Vicinal Dithiols

The recombinant polypeptides for *Chlamydomonas* LC14 and LC16 and outer arm dynein were specifically bound to PAO-agarose and eluted from a resin with 2-mercaptoethanol (Patel-King *et al.*, 1996). Thus, they may contain vicinal dithiol competent to undergo reversible oxidation/reduction. From the sequence similarity between LC14 and LC16 and the TRXR region of IC1, we investigated whether the recombinant polypeptide for the TRXR region of IC1 (Figure 9A) and outer arm dynein (Figure 9B) are bound to PAO-agarose resin. When bacterial cell lysates were applied, 80% of expressed polypeptide was recovered in the eluted fraction (Figure 9A, lane e). Although lysates contained many proteins other than recombinant polypeptide, it should be noted that the TRXR region was specifically purified by ThioBond affinity chromatography. When 21S dynein was applied, 45% of the DHC bands were recovered in the eluted fraction. The relative intensities of three IC bands did not change in the unbound (Figure 9, lane u) and eluted (Figure 9, lane e) fractions. Thus, we conclude that the sea urchin outer arm dynein IC1 contains vicinal dithiols competent to undergo reversible oxidation/reduction.

Outer Arm Dyneins from Distantly Related Species also Contains Vicinal Dithiols

In Western blotting of the outer arm dyneins from rainbow trout, *Halocynthia*, and *Mytilus*, the occur-

rence of the TRXR region in IC1 remained unclear except for *Halocynthia*. This was because our antibodies could not recognize the highly conserved sequence WCGPC in the thioredoxin family. We performed another experiment to clarify this point. If their outer arm dyneins contain vicinal dithiols derived from IC1, they would bind to PAO-agarose resin as in the case of sea urchin 21S dynein. As shown in Figure 10, 50% (rainbow trout), 45% (*Halocynthia*), and 40% (*Mytilus*) of the DHC bands were specifically eluted with 2-mercaptoethanol. Thus, these outer arm dyneins would also contain active vicinal dithiols derived from IC1 visualized with anti-NDPKR region antibodies. Multiple bands of IC1 visualized using anti-NDPKR region antibodies in trout and *Mytilus* outer arm dynein (Figure 8, lane 2) were found in the eluted fractions (Figure 10, lanes e in the bottom panel), and the relative intensities of these bands apparently did not change in the unbound (Figure 10, lane u) and eluted (Figure 10, lane e) fractions, suggesting that they were actually component (IC1) of outer arm dynein of the respective species.

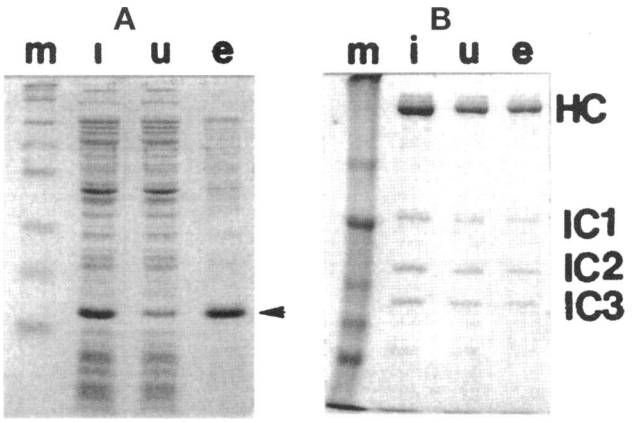

Figure 9. Affinity chromatography of the recombinant polypeptide for the TRXR region of IC1 (A) and sea urchin sperm 21S dynein (B) on PAO-conjugated agarose. (A) Transformants were cultured and induced with IPTG as described in the legend to Figure 7. The cells were collected and suspended in 50 mM phosphate buffer (pH 7.8) containing 300 mM NaCl, 1 mM DTT, and 0.1 mM PMSF. They were broken with a CRYO-PRESS (MICROTEC-NICHION, Chiba). After centrifugation, the supernatant was dialyzed overnight against TENT buffer containing 0.1 mM PMSF to remove DTT. Lane m, molecular weight markers (New England Biolabs, Beverly, MA; 16, 25, 32.5 47.5, 62, and 83 kDa from the bottom); lane i, initial fraction; lane u, unbound fraction; lane e, eluted fraction. Arrowhead indicates positions of the TRXR region of dynein IC1. (B) The 21S dynein was reduced with 1 mM DTT and dialyzed overnight to remove DTT against TENT buffer. Lane m, molecular weight markers (Sigma); lane i, initial fraction; lane u, unbound fraction; lane e, eluted fraction.

NDP Kinase Activity in the Sea Urchin Flagellar Axonemal Extract

High salt extract of sea urchin sperm axonemes was fractionated into 27 fractions by sucrose-density gradient centrifugation. An aliquot of each fraction was assayed for NDP kinase activity to locate the enzyme and subjected to Western blotting to locate the IC1 band. IC1 occurred at high S value in association with DHCs and at low S value possibly in dissociation with them. A single peak of the NDP kinase activity was detected in fractions 22–24 (a peak at fraction 23), whereas DHCs and dissociated IC1 were present in fractions 6–10 (a peak at fraction 9) and 17–21 (a peak

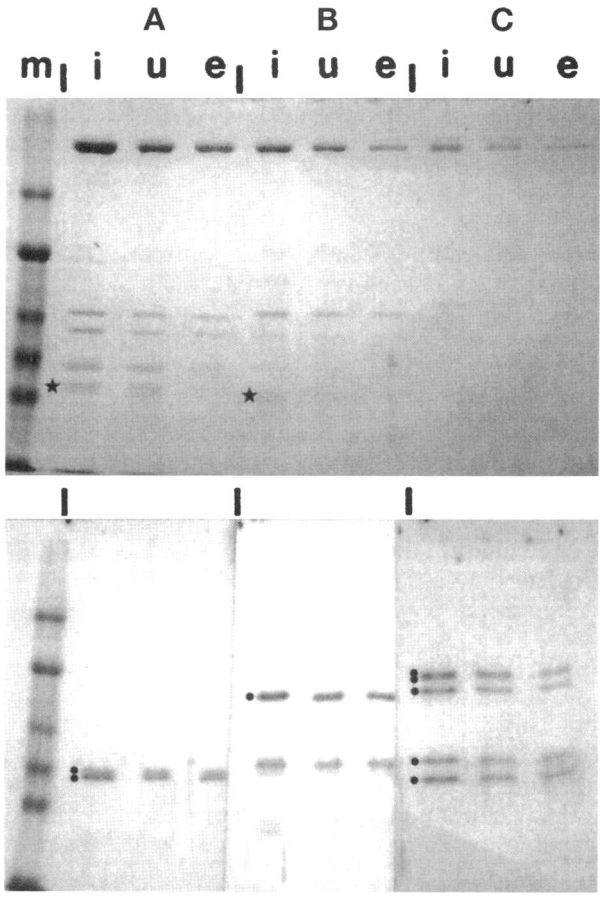

Figure 10. The PAO-agarose chromatography of the outer arm dyneins from distantly related species. Outer arm dyneins from rainbow trout (A), *Halocynthia* (B), and *Mytilus* (C) were applied to a PAO-agarose column. The initial (lane i), unbound (u), and eluted (e) fractions were subjected to 7.5% SDS-PAGE. One gel was stained with Coomassie brilliant blue (top panel) and the other was subjected to Western blotting followed by visualization using anti-NDPKR region antibodies (bottom panel). In the top panel, it should be noted that the proteins marked with the star symbol that were hardly seen in eluted fractions of rainbow trout (A) and *Halocynthia* (B). In the bottom panel, it should be noted that the numbers of bands indicated with small dots visualized using antibodies were the same between unbound (lane u) and eluted (lane e) fractions.

at fraction 20), respectively. Thus, the protein(s) responsible for the NDP kinase activity should be different from IC1 or IC1 containing dynein particle. In other words, IC1 in dynein particle or alone would not exhibit NDP kinase activity to a detectable extent.

DISCUSSION

Outer arm dynein IC1 has been implied in forming a force-generating unit with β-DHC (Sale and Fox, 1988). In the present work, we determined for the first time the cDNA sequence of the sea urchin outer arm dynein IC1. The deduced amino acid sequence of IC1 was different from IC2 and IC3, which are members of the WD family. Moreover, the sequence of IC1 suggests that it is a multifunctional protein. Thus, IC1 is a novel dynein intermediate chain.

Outer arm dynein IC1 of the sea urchin *Tripuneustes gratilla* resolves into two bands on SDS-polyacrylamide gels (Tang *et al.*, 1982). In the case of the sea urchin *A. crassispina*, IC1 seemed to be a single species (Ogawa *et al.*, 1990). However, when IC1 blotted onto an Immobilon-P membrane was stained with Coomassie brilliant blue, it appeared to resolve into two closely spaced bands. As described in RESULTS, an in-frame deletion of the sequence EVKDSVASISMEQSQ (residue numbers 632–647) was found in some clones. This variation may be due to alternative splicing resulting in different isoforms of IC1. The two bands of IC1 observed after SDS-PAGE may reflect the occurrence of different versions of mRNA for IC1. In the case of the sea urchin *A. crassispina*, the two polypeptides were minimally spaced on a polyacrylamide gel. This makes it unlikely that Northern blot (Figure 2) could resolve the two messages, especially since they are predicted to differ by 45 nucleotides.

The outer arm dynein of rainbow trout does not have intermediate chains larger than 89 kDa (Figure 8A, lane 6). Instead, two closely spaced 65-kDa bands copurified with the 89-kDa IC2 and the 79-kDa IC3 (Gatti *et al.*, 1989; King *et al.*, 1990). In the cases of sea urchin (Tang *et al.*, 1982), oyster (Wada *et al.*, 1995), and clam (Stephens and Prior, 1992 and 1995), no intermediate chains smaller than IC3 (M_r = 74,000, 82,500, or 91,000, respectively) were detected. The present result shows that the two trout 65-kDa polypeptides are immunologically related to sea urchin IC1 (Figure 8A, lane 2). It is possible that these polypeptides, or one of the two, may be an artifact of trout IC1 occurring during preparation of the outer arm dynein by endogenous protease(s). An intact IC1 may have much larger M_r. In the case of the mussel *Mytilus* IC1, multiple polypeptides are immunologically related to sea urchin IC1 (Figure 8C, lane 2). It is likely that the multiple *Mytilus* IC1 bands are derived from a much larger polypeptide (intact IC1) by endogenous protease(s). Alternatively, multiple isoforms of IC1 with different M_r may exist. To draw a conclusion, we must wait for the molecular cloning of IC1 from the trout and *Mytilus* because the possibility of alternative splicing of mRNA encoding IC1 cannot be ruled out.

The N-terminal part of IC1 was closely related to thioredoxin. Thioredoxin is a small, ubiquitous and multifunctional protein with two redox-active half-cystine residues in an exposed active center, having the WCGPC sequence (Holmgren, 1985). The well-known function of thioredoxin is a redox reaction, a dithiol-disulfide exchange reaction of a targeting protein through the reversible oxidation of its active center dithiol $(SH)_2$ to a disulfide S_2:

$$\text{thioredoxin-}(SH)_2 + \text{protein-}S_2 \rightarrow \text{thioredoxin-}S_2 + \text{protein-}(SH)_2$$

The ATPase activity of the sea urchin dynein (Ogawa and Mohri, 1972) and *Tetrahymena* dynein (Shimizu and Kimura, 1974) was biphasically affected by the addition of sulfhydryl reagents, suggesting an important role of the sulfide group(s) of dynein in its enzyme activity. It is a fascinating speculation that the TRXR part of IC1 catalyzes a reversible dithiol-disulfide exchange reaction of dynein, resulting in the regulation of dynein ATPase activity:

$$\text{IC1-}(SH)_2 + \text{DHC-}S_2 \rightarrow \text{IC1-}S_2 + \text{DHC-}(SH)_2$$

This would also imply the presence of an IC1 reductase and a reducing agent in flagella since thioredoxin and thioredoxin reductase are a powerful NADPH-protein disulfide reductase system (Holmgren, 1985).

Patel-King *et al.* (1996) showed, by sequence homology of the *Chlamydomonas* outer arm dynein LC14 and LC16, that both LC14 and LC16 are closely related to thioredoxin. These two *Chlamydomonas* LCs were proved to be redox-active since they bound to PAO-conjugated agarose that is designed for affinity purification of proteins, such as thioredoxin and thioredoxin-fusion proteins, that contain vicinal dithiols. *Chlamydomonas* outer arm dynein but not inner arm dynein could be purified on a PAO-resin by the functional vicinal dithiols of LC14 and LC16 which are associated with dynein (Patel-King *et al.*, 1996). In the sea urchin, the TRXR part of the IC1 and outer arm dynein specifically bound to a PAO-resin and eluted from the matrix with 2-mercaptoethanol as shown in Figure 9. By computational analysis, IC1 is more closely related to LC14 and LC16 than to thioredoxin and more related to LC14 than to LC16. By taking the possible functions of vicinal dithiols attributed to these polypeptides into consideration, IC1 and *Chlamydomonas* LC14 and LC16 may form a novel subfamily of thioredoxin. Although the *Chlamydomonas* outer arm dynein was previously believed not to contain polypeptides homologous to sea urchin IC1

(for a review, see Witman *et al.*, 1994), molecular clonings of IC1 and LC14/LC16 revealed that the IC1 homologues, at least in part, do exist in this species.

The NDPKR region exists in the outer arm dynein of all animal sperm examined to date. NDP kinases are enzymes of M_r 17,000 required for the synthesis of NTP other than ATP. They provide the NTPs for nucleic acid synthesis, CTP for lipid synthesis, UTP for polysaccharide synthesis, and GTP for protein elongation, signal transduction, and microtubule polymerization according to the PROSITE database (Bairoch, 1991). Yanagisawa *et al.* (1968) found the transphosphorylation reaction between GDP and ATP with digitonin-treated flagella of sea urchin sperm, and Kobayashi *et al.* (1976) investigated the NDP kinase activity of sea urchin sperm flagella and *Tetrahymena* cilia, which led to the conclusion that this enzyme associates with the outer doublet microtubules but not with the outer arm dynein. The present results also suggested that 21S outer arm dynein fraction did not show NDP kinase activity. Furthermore, IC1 alone did not exhibit the activity to a detectable extent. Thus, the roles of NDPKR sequences of IC1 remained unknown. It is an interesting feature that IC1 contains three copies of NDPKR sequences in the midregion of the polypeptide and that each copy contains a putative active site residue histidine. That IC1 has three copies of the NDPKR sequence is particularly interesting since eukaryotic NDP kinases are hexamers made of the random associations between two highly related chains, types A and B (Gilles *et al.*, 1991). If one assumes that IC1 is a novel family of NDP kinase, it may be that self-association of IC1 makes it multimeric in regard to the active sites for NDP kinase. This fact also reminds one that β-DHC, an in situ partner of IC1, also contains four copies of putative ATP-binding, possibly ATPase sites (Ogawa, 1991).

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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., and Lipman, D.J. (1990). Basic local alignment research tool. J. Mol. Biol. *215*, 403–410.

Bairoch, A. (1991). PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. *19*, 2241–2245.

Chou, P.Y., and Fasman, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. *47*, 45–148.

Eisenberg, D., Sweet, R.M., and Terwilliger, T.C. (1984). The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. USA *81*, 140–144.

Gagnon, C., White, A., Huitorel, P., and Cosson, J. (1994). A monoclonal antibody against the dynein IC1 peptide of sea urchin spermatozoa inhibits the motility of sea urchin, dinoflagellate, and human flagellar axonemes. Mol. Biol. Cell *5*, 1051–1063.

Gatti, J.-L., King, S.M., Moss, A.G., and Witman, G.M. (1989). Outer arm dynein from trout spermatozoa. Purification, polypeptide composition, and enzymatic properties. J. Biol. Chem. *264*, 11450–11457.

Gilles, A.-M., Presecan, E., Vonica, A., and Lascu, I. (1991). Nucleoside diphosphate kinase from human erythrocytes: structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. J. Biol. Chem. *266*, 8784–8789.

Holmgren, A. (1985). Thioredoxin. Annu. Rev. Biochem *54*, 237–271.

Holzbaur, E.L.F., and Johnson, K.A. (1986). Rate of ATP synthesis by dynein. Biochemistry *25*, 428–434.

Hoshi, M., Numakunai, T., and Sawada, H. (1981). Evidence for participation of sperm proteinases in fertilization of the solitary ascidian, *Halocynthia roretzi*: effects of protease inhibitors. Dev. Biol. *86*, 117–121.

Inaba, K. (1994). Cross-linking of β heavy chain subunit of dynein from sea urchin sperm flagella by dimethyl suberimidate. Biomed. Res. *15*, 123–126.

Inaba, K. (1995). ATP-dependent conformational changes of dynein: evidence for changes in the interaction of dynein heavy chain with the intermediate chain 1. J. Biochem. *117*, 903–907.

Inaba, K., and Mohri, H. (1989). Dynamic conformational changes of 21S dynein ATPase coupled with ATP hydrolysis revealed by proteolytic digestion. J. Biol. Chem. *264*, 8384–8388.

Inaba, K., and Morisawa, M. (1991). A chymotrypsin-like protease involved in motility of sperm in salmonid fish. Biomed. Res. *12*, 435–437.

Inaba, K., and Morisawa, M. (1992). Chymotrypsin-like protease activity associated with demembranated sperm of chum salmon. Biol. Cell *76*, 329–333.

King, S.M., and Witman, G.B. (1990). Localization of an intermediate chain of outer arm dynein by immunoelectron microscopy. J. Biol. Chem. *265*, 19807–19811.

King, S.M., Wilkerson, C.G., and Witman, G.B. (1991). The M_r 78,000 intermediate chain of *Chlamydomonas* outer arm dynein interacts with α-tubulin in situ. J. Biol. Chem. *266*, 8401–8407.

King, S.M., Gatti, J.L., Moss, A.G., and Witman, G.B. (1990). Outer-arm dynein from trout spermatozoa: substructural organization. Cell Motil. Cytoskel. *16*, 266–278.

King, S.M., Patel-King, R.S., Wilkerson, C.G., and Witman, G.B. (1995). The 78,000-M_r intermediate chain of *Chlamydomonas* outer arm dynein is a microtubule-binding protein. J. Cell Biol. *131*, 399–409.

Kobayashi, T., Kaji, K., and Sakai, H. (1976). Nucleoside diphosphate kinase and the flagellar movement of glycerinated spermatozoa. J. Biochem. *79*, 413–418.

Kodama, T., Fukui, K., and Kometani, K. (1986). The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified malachite green method for determination of inorganic phosphate. J. Biochem. *99*, 1465–1472.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Mierendorf, R.C., Percy, C., and Young, R.A. (1989). Gene isolation by screening λgt11 libraries with antibodies. Methods Enzymol. *152*, 458–469.

Mitchell, D.R., and Kang, Y. (1991). Identification of *oda6* as a *Chlamydomonas* dynein mutant by rescue with the wild-type gene. J. Cell Biol. *113*, 835–842.

Mitchell, D.R., and Kang, Y. (1993). Reversion analysis of dynein intermediate chain function. J. Cell Sci. *105*, 1069–1078.

Moss, A.G., Sale, W.S., Fox, L.A., and Witman, G.B. (1992). The α subunit of sea urchin sperm outer arm dynein mediates structural and rigor binding to microtubules. J. Cell Biol. *118*, 1189–1200.

Neer, E.J., Schmidt, C.J., Nambudripad, R., and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature *371*, 297–300.

Ogawa, K. (1991). Four ATP-binding sites in the midregion of the β heavy chain of dynein. Nature *352*, 643–645.

Ogawa, K. (1992). Primary structure and function of a dynein motor molecule. Zool. Sci. *9*, 265–274.

Ogawa, K., and Mohri, H. (1972). Studies on flagellar ATPase from sea urchin spermatozoa. I. Purification and some properties of the enzyme. Biochim. Biophys. Acta *256*, 142–155.

Ogawa, K., Yokota, E., and Shirai, H. (1988). Assembly of a protein sharing epitopes with sperm dynein heavy chains into meiotic spindle in the prometaphase starfish oocyte. Biol. Cell *64*, 57–66.

Ogawa, K., Kamiya, R., Wilkerson, C.G., and Witman, G. (1995). Interspecies conservation of outer arm dynein intermediate chain sequences defines two intermediate chain subclasses. Mol. Biol. Cell *6*, 685–696.

Ogawa, K., Yokota, E., Hamada, Y., Wada, S., Okuno, M., and Nakajima, Y. (1990). The outer arm dynein α-heavy chains of sea urchin sperm flagella and embryonic cilia are different. Cell Motil. Cytoskel. *16*, 58–67.

Patel-King, R.S., Benashski, S.E., Harrison, A., and King, S.M. (1996). Two functional thioredoxins containing redox-sensitive vicinal dithiols from the *Chlamydomonas* outer dynein arm. J. Biol. Chem. *271*, 6283–6291.

Porter, M.E., and Johnson, K.A. (1983). Characterization of the ATP-sensitive binding of *Tetrahymena* 30 S dynein to bovine brain microtubules. J. Biol. Chem. *258*, 6575–6581.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sale, W.S., and Fox, L.A. (1988). Isolated β-heavy chain subunit of dynein translocates microtubules in vitro. J. Cell Biol. *107*, 1793–1797.

Shimizu, T. (1987). The substrate specificity of dynein from *Tetrahymena* cilia. J. Biochem. *102*, 1159–1165.

Shimizu, T., and Furusawa, K. (1986). Phosphorothioate analogues of adenosine 5′- triphosphate as substrates of dynein from *Tetrahymena* cilia. Biochemistry *25*, 5787–5792.

Shimizu, T., and Kimura, I. (1974). Effects of N-ethylmaleimide on dynein adenosine triphosphatase activity and its recombining ability with outer fibers. J. Biochem. *76*, 1001–1008.

Stephens, R.E., and Prior, G. (1992). Dynein from serotonin-activated cilia and flagella: extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. J. Cell Sci. *103*, 999-1012.

Stephens, R.E., and Prior, G. (1995). Dynein inner arm heavy chain identification in cAMP- activated flagella using class-specific polyclonal antibodies. Cell Motil. Cytoskel. *30*, 261–271.

Tang, W.-J.Y., Bell, C.W., Sale, W.S., and Gibbons, I.R. (1982). Structure of the dynein-1 outer arm in sea urchin sperm flagella. I. Analysis by separation of subunits. J. Biol. Chem. *257*, 508–515.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.

Vallee, R. (1993). Molecular analysis of the microtubule motor dynein. Proc. Natl. Acad. Sci. USA *90*, 8769–8772.

Wada, S., Okuno, M., Nakamura, K.-I., and Mohri, H. (1992). Dynein of sperm flagella of oyster belonging to *Protostomia* also has a two-headed structure. Biol. Cell *76*, 311–317.

Witman, G.B., Wilkerson, C.G., and King, S.M. (1994). The biochemistry, genetics and molecular biology of flagellar dynein. In: Microtubules, ed. J.S. Hyams and C.W. Lloyd, New York: Wiley-Liss, 229–249.

Yanagisawa, T., Hasegawa, S., and Mohri, H. (1968). The bound nucleotides of the isolated microtubules of sea-urchin sperm flagella and their possible role in flagellar movement. Exp. Cell Res. *52*, 86–100.