doi:10.1006/jmbi.2001.4566 available online at http://www.idealibrary.com on IDEAL® *J. Mol. Biol.* (2001) **307**, 1317–1327

JMB

Antibodies to Cytoplasmic Dynein Heavy Chain Map the Surface and Inhibit Motility

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Polyclonal antibodies have been raised against four 16 residue peptides with sequences taken from the C-terminal quarter of the human cytoplasmic dynein heavy chain. The sites are downstream from a known microtubule-binding domain associated with the ''stalk'' that protrudes from the motor domain. The antisera were assayed using bacterially expressed proteins with amino acid sequences taken from the human cytoplasmic dynein heavy chain. Every antiserum reacted specifically with the appropriate expressed protein and with pig brain cytoplasmic dynein, whether the protein molecules were denatured on Western blots or were in a folded state. But, whereas three of the four antisera recognized freshly purified cytoplasmic dynein, the fourth reacted only with dynein that had been allowed to denature a little. After affinity purification against the expressed domains, whole IgG molecules and Fab fragments were assayed for their effect on dynein activity in *in vitro* microtubule-sliding assays. Of the three anti-peptides that reacted with fresh dynein, one inhibited motility but the others did not. The way these peptides are exposed on the surface is compatible with a model whereby the dynein motor domain is constructed from a ring of AAA protein modules, with the C-terminal module positioned on the surface that interacts with microtubules. We have tentatively identified an additional AAA module in the dynein heavy chain sequence, which would be consistent with a heptameric ring.

Keywords: motility; microtubules; video-microscopy; electron microscopy; AAA proteins

Introduction

Cytoplasmic dynein (CD) is a multisubunit protein that uses ATP to translocate along microtubules (Holzbaur & Vallee, 1994). It has roles in cytoplasmic vesicle motility and, during mitosis, in chromosome separation and spindle organization (Schroer, 1994; Vallee & Sheetz, 1996). Dynein purified from brain tissues is a homodimer of ~500 kDa heavy chains (CDHCs) together with two intermediate chains (CDICs) of 70-80 kDa, four light intermediate chains (CDLICs) of 50-60 kDa and various light chains (CDLCs) (Holzbaur & Vallee, 1994). This complex drives microtubule sliding *in vitro* (Vallee & Sheetz, 1996). The dynactin protein complex that co-purifies with CD (Gill *et al*., 1991; Schroer & Sheetz, 1991) seems to be needed for most of its *in vivo* activities (Karki & Holzbaur, 1999) but, *in vitro*, recombinant CDHC can by itself produce microtubule gliding with a velocity similar to that of native dynein (Mazumdar *et al*., 1996).

The N-terminal 1200-1300 residues dimerise as a stem domain that interacts with CDIC and tethers dynein to its cargo (Habura *et al*., 1999; Lyadurai *et al*., 1999). The remaining ~380 kDa form the globular catalytic head domain, responsible for direct interaction with microtubules, as well as for ATP hydrolysis and force production (Vallee, 1993). The central region contains four phosphate-binding loop (P-loop) motifs (Gee & Vallee, 1998; Vallee, 1993), each part of a *bona fide* nucleotide-binding site (Mocz *et al*., 1998). However, ATPase activity is lost if the first P-loop is truncated or if the polypeptide is cleaved there by UV light in the presence of vanadate (Lee-Eiford *et al*., 1986). The head domain has been expressed without the stem in two systems (Gee *et al*., 1997; Koonce & Samso,

Abbreviations used: CD, cytoplasmic dynein; CDHC, CD heavy chain; CDIC, CD intermediate chain; CDLIC, CD light intermediate chain; CDLC, CD light chain.

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0022-2836/01/051317–11 $35.00/0

1996; Koonce, 1997) and shown to bind microtubules in an ATP-sensitive fashion and to undergo UV-vanadate cleavage. Any further truncation affects ATP binding or ATPase activity, though some microtubule binding persists if all four P-loops are removed or if ~1170 residues are cut from the C terminus. A ~300 residue region after the last P-loop includes two alpha-helical segments separated by a small globular domain. Gee & Vallee (1998) found that an expressed domain consisting only of this region was able to bind well to microtubules; in electron microscope images, shadowed specimens resembled the stalk that has been seen to extend from intact heads of both cytoplasmic and axonemal dyneins and to bind to axonemal microtubules *in situ* (Goodenough & Heuser, 1989; Burgess, 1995). Experiments by Koonce (1997) support the existence of binding activity in this region but indicate that regions downstream of the stalk sequence may also have some affinity for microtubules.

A recent search of the sequence database (Neuwald *et al*., 1999) using a reference set of sequences in the AAA ATPase family (Langer, 2000; Patel & Latterich, 1998) revealed the presence of six copies of the AAA module within the dynein motor domain sequence (see Figure 1), leading to the current model (Vale, 2000; King, 2000) of the dynein motor domain as a ring of connected subunits. Each of the four P-loops is associated with an AAA module but the analysis (Neuwald *et al*., 1999) identified two additional modules in which P-loops are no longer recognizable as such. Here, we present evidence for a seventh module, also lacking a P-loop, which correlates with electron microscopic images that show each individual dynein head as a ring of at least seven globular lobes arranged around a central cavity (Samso *et al*., 1998). The modules without P-loops are highly conserved in sequence, despite having no known function. We have made antibodies to sequences that are found within the final two modules and are predicted to lie on an exposed surface. An investigation of their properties adds new details to the model of the motor domain and suggests that at least one of the AAA modules lacking a P-loop may be involved in interacting with microtubules.

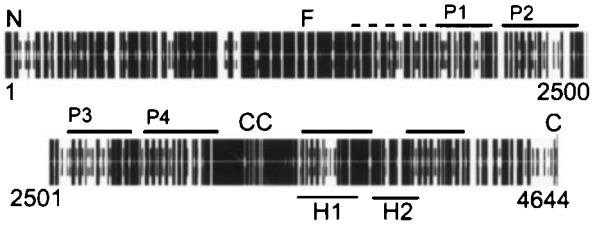

Figure 1. Plan of the cytoplasmic dynein heavy chain (CDHC). The predicted secondary structures (Rost & Sander, 1993) of the N-terminal and C-terminal halves of the rat CDHC sequence (Mikami *et al*., 1993; Zhang *et al*., 1993) are represented by vertical bars: alpha-helical regions as long bars, beta-sheet regions as shorter bars, random coil as gaps. Each half contains two P-loops (P1-P4). Removal of the N-terminal fragment (N to F) produces the minimal motor domain. Regions corresponding to expressed proteins H1 (3537-3802) and H2 (3870-4071) are indicated. Continuous horizontal lines above the secondary structure mark the six proposed AAA domains (Neuwald *et al*., 1999). The broken line marks a possible seventh globular domain. The two long helical regions before H1 are thought to form an antiparallel coiled-coil (Gee *et al*., 1997). Prediction carried out *via* http://pbil.ibcp.fr/NPSA.

Results

Expressed proteins and synthetic peptides

Since previous work suggested that it can be difficult to elicit good antibodies to the dynein heavy chain (Criswell *et al*., 1996; Gönczy *et al*., 1999; Kandl *et al*., 1995; Li *et al*., 1994; Vaisberg *et al*., 1993, 1996), we used peptides as antigens and expressed proteins for affinity purification. The criterion for choosing the peptides was that they should be soluble and hydrophilic, both for ease of handling and because amino acid sequences with such properties are likely to lie on the surface of a large molecule. Because the full sequence of human CDHC is unknown, we refer to residues numbered from the start of the full rat sequence. Clone KIAA0325, the C-terminal half of human CDHC has only 32 differences (26 of which are conservative changes) from rat residues 2558-4644, with no gaps or insertions. Most of the substitutions, including four of the six dissimilar changes, are in the last 400 residues, beyond the proposed AAA domains (Figure 1) or in between the last two AAA domains. The expressed fragment H1 of human CDHC is identical with the rat sequence, while H2 has eight conserved differences between the two species. Peptides Pt1 and Pt2 lie within H1, Pt3 and Pt4 within H2. Pt3 includes two residues that vary between rat and human, the other three are fully conserved.

Isolation of pig brain cytoplasmic dynein

Cytoplasmic dynein was prepared from pig brain by extracting a pellet of Taxol-stabilised microtubules with Mg-ATP (Amos, 1989), followed by sucrose-gradient fractionation to separate dynein from other proteins that associate with microtubules in an ATP-dependent manner (Paschal *et al*., 1987, 1993). Figure 2 shows fractions from a 5%-20% (w/v) sucrose density-gradient in Tris/KCl buffer. Two peaks gave high molecular mass bands on SDS-PAGE (Figure 2(a)) but immuno-analysis with anti-dynein antibodies, against both CDHC and CDIC (Figure 2(b)), showed that only the second peak contained dynein. As expected, dynactin comigrates with dynein under these conditions. High molecular mass proteins in the upper peak (fractions 2-5) were heat-resistant and did not react with any of the antibodies used

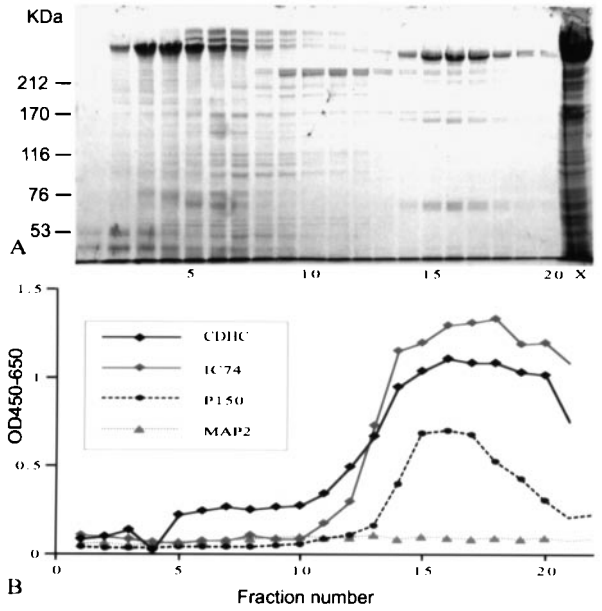

Figure 2. Pig brain dynein extracts fractionated on a 5%-20% (w/v) sucrose gradient. (a) Fractions 1-20 (in 5%-17% sucrose) on an SDS/6% polyacrylamide gel, stained with PAGE blue. X is the ATP extract loaded onto the gradient. Bands on the gels were identified by Western blotting (Figure 4(a)). (b) Immuno-analysis of the fractions in microtitre plates. Each fraction was reacted with antibodies to CD intermediate chain (IC74), to dynactin heavy chain (P150) and to MAP2, as well as with anti-Pt4 (to CDHC). Dynein and dynactin both peak in fractions 15-17 in this buffer. Dynactin p50 is present also (Figure 4(a)). The independent high molecular mass bands in fractions 1-5 are probably MAP1a,b. MAP2 is absent throughout.

in this immuno-assay, including anti-MAP2 (Sigma). The result of N-terminal protein sequencing indicated that they might be MAP1a or MAP1b.

Characterization of antisera

Rabbit antisera, collected 14 days after the second boost, were tested by ELISA against H1 and H2 (Figure 3(a) and (b) and against pig brain dynein (Figure 3(c)). Anti-Pt4 showed extremely high affinity to native dynein, anti-Pt2 and anti-Pt1 showed somewhat lower affinities. Anti-Pt3 showed very low affinity to fresh dynein but reacted as strongly as anti-Pt4 with dynein that had been left at 4 °C for two weeks (Figure 3(c)). The incubation period did not increase the reactivity of dynein with the other three antisera. The antisera to Pt1, Pt2 and Pt4 were subjected to antigen affinity purification on Sepharose 4B resin coupled to H1 or H2. Antibodies eluted with glycine are anti-Pt1G, anti-Pt2G and anti-Pt4G; those eluted with triethylamine are anti-Pt2T and anti-Pt4T. Figure 4(c) demonstrates that anti-Pt4G, anti-Pt1G, anti-Pt2T and anti-Pt2G interacted specifi-

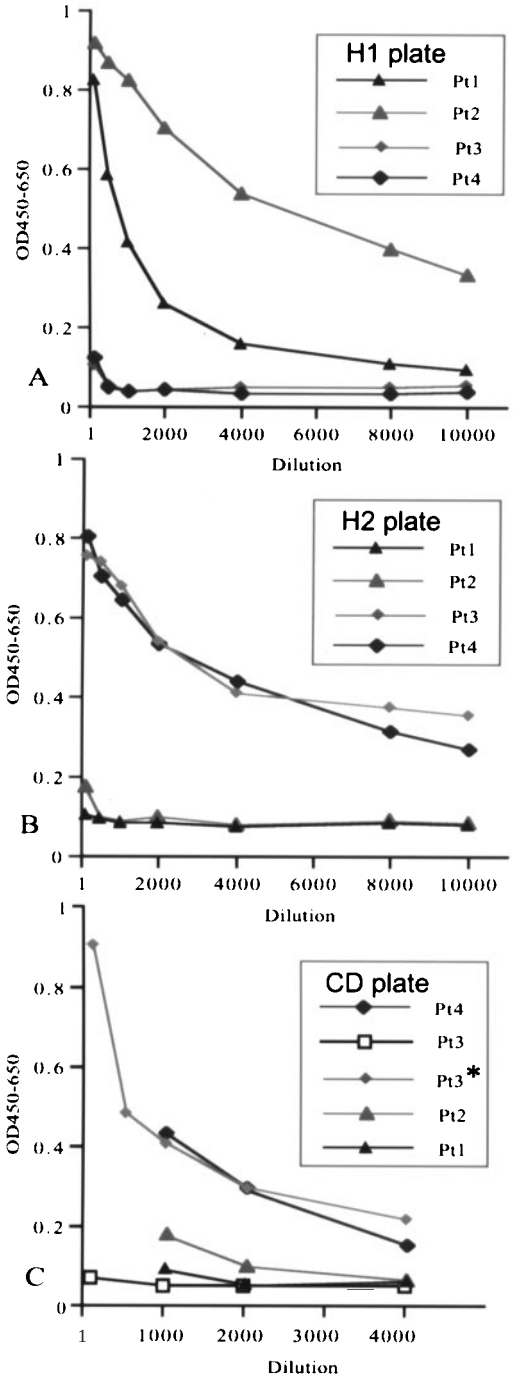

Figure 3. Immuno-assay of serially diluted final antisera against folded proteins. (a) and (b) The wells of microtitre plates were coated with expressed proteins H1 or H2. (c) Pooled fractions 15-17 of the sucrose gradient (Figure 2) were used to coat the wells. Most curves show the response to the fresh preparation. After storage of the dynein for two weeks, the responses of anti-Pt1, anti-Pt2 and anti-Pt4 were similar but the anti-Pt3 serum then reacted more strongly (*). The secondary antibody used was HRP-conjugated anti-rabbit IgG (Sigma).

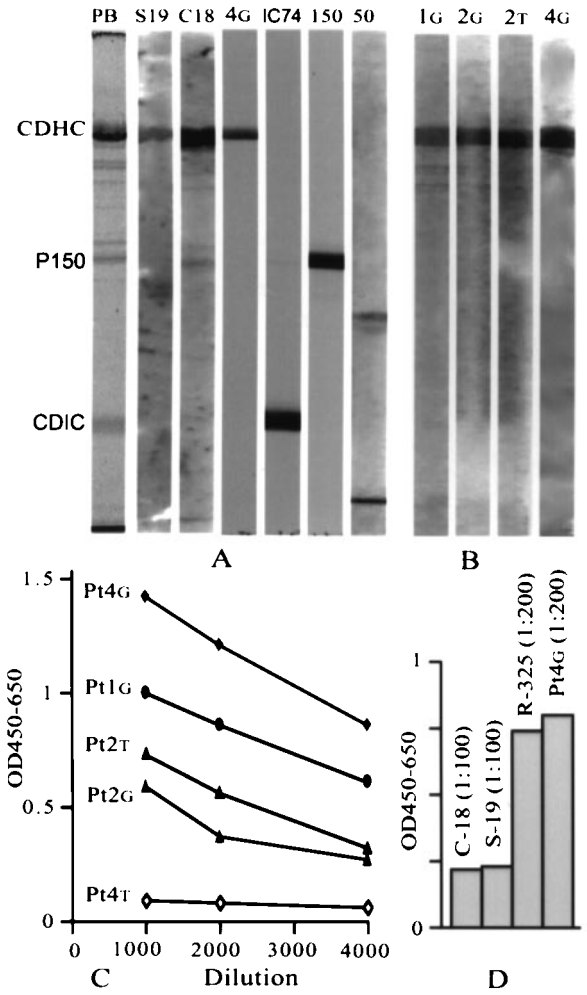

Figure 4. Immuno-analysis of fractions 15-17 of the sucrose gradient. (a) and (b) Western blotting of SDS/polyacrylamide gels. Lane 1 in (a) shows a 6% gel stained with PAGE blue. Lanes 2-4 show reactions with anti-CDHC antibodies; C-18 and S19 (Sigma) are directed against the C and N terminus, respectively. 4G is the affinity-purified anti-Pt4 fraction released by glycine. Antibody IC74.1 (in lane 5) recognises the N-terminal 60 residues of the 74 kDa intermediate chain. Lanes 6 and 7 (150 & 50) show detection of dynactin P150 and P50 polypeptides. (b) The reactions of various fractions of affinity-purified anti-peptide antibodies to CDHC. (c) ELISA assays of affinity-purified antibody fractions (see the text), serially diluted into microtitre plate wells coated with fraction 16. The starting concentration of IgG was 0.5 mg/ml. (d) Comparison of the reactions of different antibodies with fresh cytoplasmic dynein. Control antibodies C-18 and S-19 do not react well with folded CDHC. R-325 (Santa Cruz Biotechnology, Inc.) reacts as well as our anti-Pt4G but has no effect on motility.

cally and strongly with native dynein molecules, whereas anti-Pt4T failed to react. The four good affinity-purified antibody fractions (0.1-0.2 mg/ml) could all be diluted to 1:4000. Western blotting further proved that these antibodies cross-reacted specifically with the ~500 kDa CDHC band (Figure 4(b)).

Effect of antibodies on motility *in vitro*

Video-enhanced differential interference contrast (VE-DIC) microscopy showed that the purified dynein could move microtubules in the presence of Mg-ATP. Figure 5 illustrates microtubules sliding over a coverslip coated with approximately 0.2 mg/ml of freshly prepared CD. Microtubule gliding occurred in a continuous unidirectional fashion, and lasted for as long as ten hours in undisturbed preparations. A typical sliding velocity measurement was 0.46(±0.22) μm/second. An estimate of the amount of dynein bound, made by comparing the intensities of stained bands on an SDS/polyacrylamide gel, of a drop of protein before and after the five minute incubation period (Vallee & Sheetz, 1996), indicated that ~80% stuck to the surface. After the dynein-coated coverslip was incubated with anti-Pt4G instead of PBS/motility buffer, microtubule sliding resembled the effect of using a much lower concentration of dynein. Very few microtubules were seen on the surface of the coverslip. In contrast, antibodies anti-Pt1G, anti-Pt2G,T and R-325 had no obvious effect on motility; microtubules were seen gliding as smoothly and continuously as in the control. The inhibitory effect of anti-Pt4G was not due to cross-linking of dynein molecules by IgG. Anti-Pt4G Fab was even more effective than anti-Pt4G IgG, probably because of being at a higher molar concentration. A total of 1 μg in 10μl of antibody was applied for each motility assay. Thus, up to 1 μg of IgG or Fab could react with ~1.6 μg (~80% of 2 μg) of dynein on the surface; a molar ratio of ~6:1 (IgG to dynein) or ~18:1 (Fab to dynein). After treatment with this concentration of anti-Pt4G Fab, most of the surface was empty of microtubules, though in small areas (e.g. see Figure 6) a few microtubules attached and moved briefly before detaching and returning to the medium. The short movements occurred at normal sliding velocity (measured as 0.48(±0.22) μm/second).

Discussion

Peptides and antibodies

We have produced four high-affinity antibodies to different regions of the dynein heavy chain sequence (Figures 7 and 8), using peptides as antigens. Previous work (Criswell *et al.*, 1996; Gönczy *et al.*, 1999; Kandl *et al.*, 1995; Li *et al.*, 1994; Vaisberg *et al.*, 1993, 1996) has shown that it can be difficult to elicit antibodies to the dynein heavy chain but the strategy used here proved to be very successful. The original criterion for choosing the antigenic sites was that the peptides should be predicted to be soluble and hydrophilic (Hopp & Woods, 1981; Wilkinson & Harrison, 1991), both for ease of handling and because amino acid

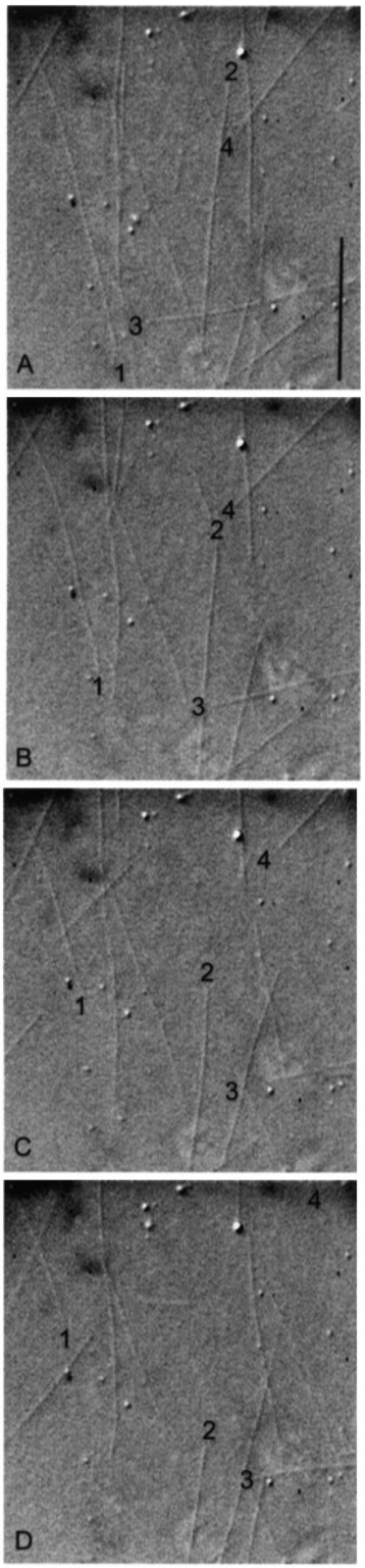

Figure 5. Microtubules sliding produced by cytoplasmic dynein. The coverslip surface was coated with fraction 16 of the sucrose gradient (Figure 2) under control conditions. Microtubules, including those labelled 1-4, moved continuously across the field. There were six second intervals between the frames shown. The scale bar represents, 10 μm.

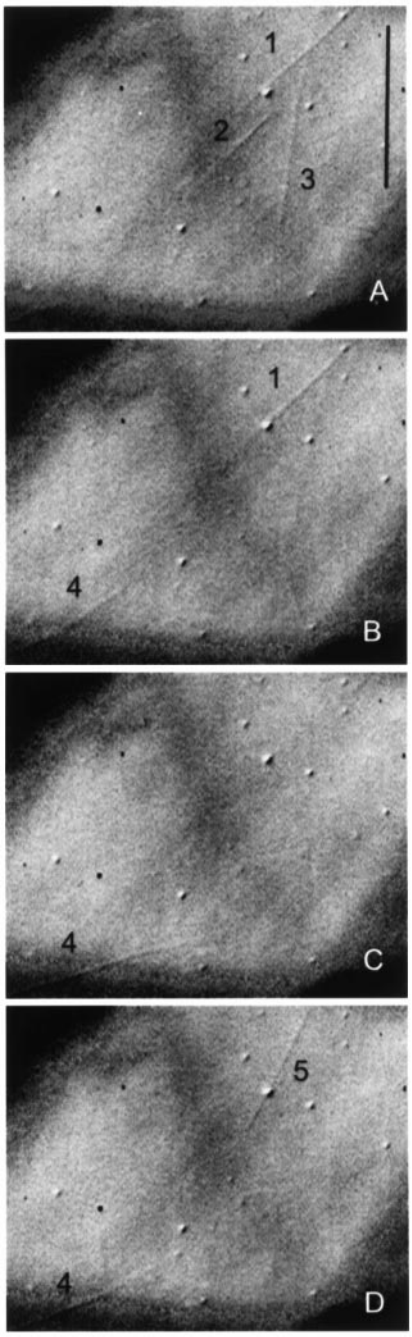

Figure 6. Effect of anti-peptide 4 on motility. Motility assay after incubation of a dynein-coated surface with Fab fragments of anti-Pt4G. Microtubules 1-5 attached to the surface only briefly. No. 4 managed to slide in a wavering path for a short time. There were six second intervals between the frames shown. The scale bar represents 10 μm.

sequences with such properties are likely to lie on the surface of a large molecule. All four antisera were found to interact specifically with the appropriate expressed proteins in a folded state and three interacted in a specific manner with wholly intact cytoplasmic dynein molecules. After affinity

Figure 7 (*legend shown opposite*)

purification against expressed proteins corresponding to segments of CDHC, anti-Pt4G and anti-Pt1G showed the highest affinities for native dynein but even anti-Pt2T and anti-Pt1G could detect dynein after dilution to ~0.1 μg/ml (Figure 4). Anti-Pt3 interacted with folded H1 and with ''old'' whole dynein, though not with freshly isolated native protein. This suggests that Pt3 lies on a part of the surface that is accessible to antibodies only when the molecule loses some aspect of its native conformation. Freshly isolated cytoplasmic dynein usually appears quite compact, with its two motor domains (heads) in close association (Amos, 1989), as shown by the examples of similar images obtained recently (Figure 8(c)). In samples that are no longer active in motility assays, the heads tend to separate and appear more disordered.

Only one previous report, using antibodies directed against the region containing the first two P-loops of CDHC, has demonstrated inhibition of motility; however, it required the application *in vitro* of 8-12 mg/ml of this antibody (Vaisberg *et al*., 1993). It is not clear how specific the interaction would be at such high concentrations. We therefore used a lower antibody concentration, 0.1 mg/ml, throughout our experiments. Most of the antibodies we tried in motility assays had no effect on the interaction between fresh dynein and microtubules; microtubule sliding continued as in untreated specimens. However, one antibody had a marked effect on binding and motility at this concentration. Very few microtubules were able to bind to a glass surface that had been coated with dynein and then treated with anti-Pt4G. Any microtubules that bound to and slid over the surface did so for only brief periods, as if interacting with dynein at very low surface density. The most likely explanation is that anti-Pt4G binds to a site on dynein where it directly blocks the dynein-microtubule interaction. It is unlikely, for example, that binding of antibody causes dynein to release from the glass surface, since control molecules were attached firmly enough to sustain microtubule sliding for an hour or more. The Pt4 antigenic site may form part of the tubulin-biding interface or must at least lie very close by. In contrast, anti-Pt1 and anti-Pt2 recognised fresh dynein on ELISA plates but had no detectable effect on motility. They presumably bind to sites on the molecule where they do not interfere with its interactions with microtubules.

Motor domain as a ring of AAA subunits

The minimal motor domain seen by EM (Samso *et al*., 1998) has the appearance of a ring of at least seven subunits. It has been proposed (Vale, 2000) that the AAA protein modules predicted by Neuwald *et al*. (1999) correspond to six subunits. The alignment of sequences within the proposed AAA modules is illustrated for mammalian CDHC in Figure 7. Four modules include the obvious P-loops but a further two modules without P-loops lie on the other side of the stalk region (see Figure 8(a)). Moreover, secondary structure predictions (Figure 1) are consistent with the existence of a seventh module (S0) upstream of the first P-loop. Since there is also some sequence homology, at least in the region of the Walker A and Walker B motifs (Figure 7), it is possible that the structural fold of S0 also resembles an AAA protein. The region at the C terminus that is not yet accounted for is also large enough to accommodate a separate subunit but the near-absence of predicted beta-sheet in this region suggests a distinctly different fold from that of S1-S6.

As can be seen in Figure 7, three of the peptides we chose as antigens (Pt1, Pt2, and Pt4) lie within AAA modules S5 or S6. The antibodies to these peptides all interact with intact native dynein. In the structure of the *Escherichia coli* heat-shock protein HSLU, a complete ring complex of AAA proteins that has been solved to atomic resolution (Bochtler *et al*., 2000), the regions corresponding to Pt1 and Pt4 lie on the outer surface of the complex. In the case of Pt2, the dynein sequence has an extra loop adjacent to a piece of sequence that is exposed on HSLU. These results are fully consistent with the postulate that the dynein motor domain is a ring of linked modules, each with a fold homologous to an AAA protein. Pt3 occurs in the linker sequence between subunits S5 and S6, and must lie somewhere where it is protected from antibodies when dynein is in its compact conformation. Possible sites include those between the two heads, on the inside of the ring of subunits or between subunits. The Pt3 region may be less important functionally than the other three peptides, since its sequence is more divergent among different species.

Figure 7. Rat cytoplasmic dynein heavy chain sequence (residues 1874-4319). Segments labelled Box II-VIII, Walker A, B or Motif C (residues in red or blue) were identified by their homology to other members of the AAA-ATPase family (Neuwald *et al*., 1999). The sequence was originally shown to contain six such domains in succession (S1-S6). Residues in red are identical with those in many other members of the family. Variable loops between the conserved segments of the AAA family are shown in black; the final set of sequences in black presumably connects neighbouring ''subunits''. Sequences in the first part of a possible subunit preceding S1 (referred to here as S0) are shown for three distant species (rat, *Dictyostelium* and *Saccharomyces cerevisiae*). Peptides used here as antigens against mammalian CDHC are shown in green; Pt1 is in Box VII′ of S5, Pt2 is just before Box VII'' of S5, Pt3 comes after S5, Pt4 covers most of the Walker-A region of S6. The sequences of expressed proteins H1 and H2 are shown underlined.

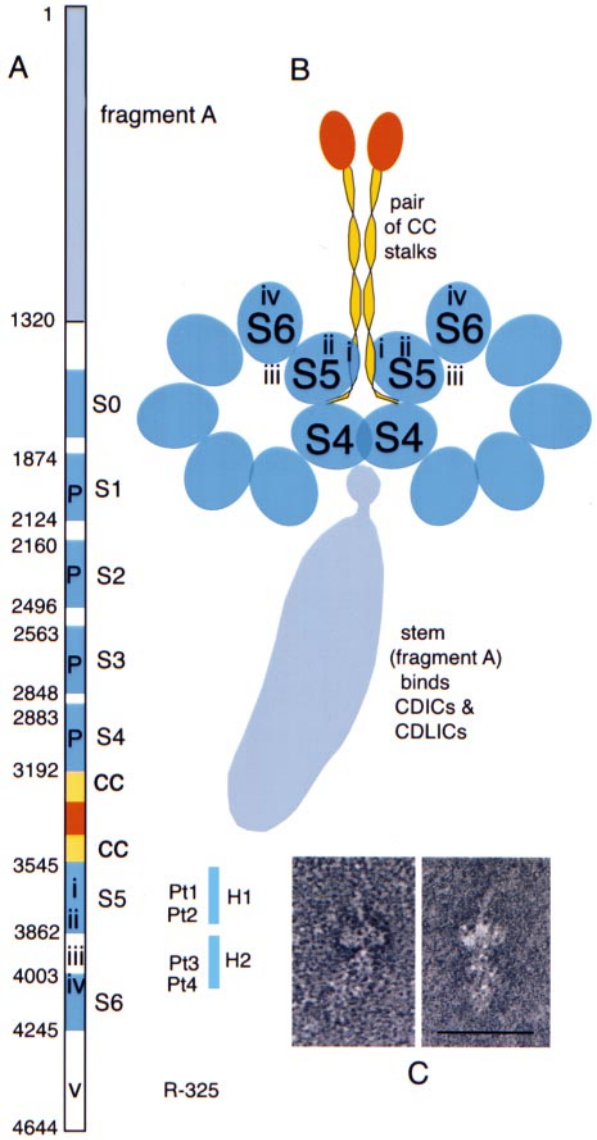

Figure 8. Structure of cytoplasmic dynein heavy chain. (a) Plan of the major domains of CDHC. The numbers refer to the rat amino acid sequence. Fragment A can be removed without affecting ATPase activity or ATP-dependent microtubule binding (Gee & Vallee, 1998). Subunits S1-S6 were identified by sequence homology as AAA modules (Neuwald *et al*., 1999). S0 is a proposed seventh subunit (see Figure 7). The antiparallel coiled-coil (CC) domain lies between S4 and S5. Numbers i to iv in S5-S6 indicate the positions of the four peptides (Pt1-Pt4) used to raise antibodies in the present work. Antibody v (R-325, Santa Cruz) was raised against C-terminal residues 4320-4644. (b) Tentative diagrammatic model of a cytoplasmic dynein molecule, which consists of two heavy chains dimerised *via* the N-terminal parts of the heavy chains and their associated intermediate (CDICs) and light intermediate chains (CDLICs). The coiled-coil stalks also appear to dimerise under some conditions. The exact relationship between the two heads is not known, nor the relative arrangement of the globular AAA domains in each head but a tentative placing of subunits 4-6 is shown. The order of the remaining subunits is unknown. Since there is an extensive stretch of amino acid sequence between the stem domain and S0, the first subunit of the heptamer, the latter may be positioned far from the top of the stem. (c) Electron microscope images of two individual dynein molecules freshly isolated from pig brain, contrasted in negative stain. The scale bar represents 50 nm.

Dynein-microtubule interface

In experiments with expressed proteins, microtubule-binding activity was found in the C-terminal half of the sequence, after the four P-loops (Gee *et al*., 1997; Koonce, 1997; Koonce & Tikhonenko, 2000). Gee *et al*. narrowed down the binding activity to just the loop between the two long helical regions. However, some parts of the main surface of the globular head may also make contact with tubulin during the ATPase cycle. Only the globular tip of the coiled-coil stalk has been clearly observed to interact with microtubules by electron microscopy (Goodenough & Heuser, 1989; Burgess, 1995) but the images obtained probably represent particularly stable stages in the enzymatic cycle. It seems unlikely that a dynein head can move distances as large as 12 nm (Burgess, 1995) simply by tilting around the tip of the stalk. A possible role for subunits that lack ATPase activity is to make transient contact with tubulin. Koonce (1997) found that a construct corresponding to residues 3678-3818 of *Dictyostelium* dynein (residues 3596-3736 of rat CDHC), downstream of the stalk domain, bound weakly to microtubules. This result suggests that part of subunit S5 may interact directly with tubulin. Since the end of this latter region overlaps with Pt1, it is slightly surprising that anti-Pt1 did not interfere with microtubule sliding, especially since Pt1 shows high similarity even between cytoplasmic and axonemal dyneins. An alternative explanation for the effect of anti-Pt4 is that it has an indirect effect and, by binding to S6, affects the conformation of the motor domain elsewhere; in this case, S6 would not need to be at or close to the tubulin-binding interface.

However, results implicate the involvement of subunit S6 in the interaction. Not only do antibodies to Pt4 prevent microtubules from attaching to dynein coated glass but we have also found that our expressed protein H2 can be pelleted with microtubules (our unpublished results). Pt4 is less conserved in sequence than Pt1 but the charged and hydrophobic residues are mostly conserved. Thus, in a preliminary scheme for the layout of CDHC (Figure 8(b)), we have tentatively put S6 near the top of the head, close to the free end of the stalk, where this part of the surface would be in a position to interact with the next microtubule. We hope to substantiate this by immuno-electron microscopy to identify the site of Pt4 on the dynein molecule.

Materials and Methods

Protein cloning, expression and purification

The KIAA0235 plasmid (Kazusa DNA Research Institute, Japan) subcloned into pBlueScriptII KS, served as the template for cloning and expression of two fragments, H1 and H2 (Figure 1) into vector pQE system (Qiagen). The fragments were chosen to have a high hydrophilicity index (Hopp & Woods, 1981) and a low insolubility factor (Wilkinson & Harrison, 1991), using the program DNAid (freeware, F. Dardel, E-mail: fred@botrytis.polytechnique.fr).

Details of cell culture and harvesting will be described elsewhere. Inclusion body pellets containing H1 were prepared by standard protocols (Sambrook *et al*., 1989) and resuspended in Ni-column binding buffer (100 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM Tris-HCl, 1 mM PMSF (phenylmethyl-sulfonylfluoride), 10 mM imidazole, 2.5 mM β-mercaptoethanol). H1 was denatured in 8 M urea and loaded onto a Ni-NTA column. Proteins bound to the column were refolded there at room temperature with a gradient of decreasing urea and increasing β-mercaptoethanol and then eluted with a gradient of 20 mM to 500 mM imidazole in native elution buffer (50 mM sodium phosphate (pH 7.2), 300 mM NaCl, 0.1 mM PMSF, 5 mM β-mercaptoethanol and 10 % glycerol) at 4 °C. H2, loaded under native conditions at 4 °C on a 10 ml Ni-NTA Superflow column, was eluted with a linear gradient of 20 mM to 500 mM imidazole in native elution buffer.

Production of antipeptide antisera

Peptides to be used as antigens were synthesized with an additional C-terminal cysteine residue by Peptide Products Ltd. (Oldham, UK). Their sequences were predicted to be soluble and hydrophilic: Pt1, VLKAERPDVDEKRSDLC; Pt2, LENLKREAAEVTRKVEC; Pt3, FLRGNEIVLSAGSTPRC; Pt4, VPGYDASGHVEDLAAEC.

Each peptide was coupled to the carrier protein, keyhole limpet hemocyanin (KLH), through the C-terminal cysteine residue, using 3-maleimidibenzoyl-*N*-hydroxysuccinimide (MBS) as coupling reagent (Kitagawa, 1976; Liu *et al*., 1979) according to the manufacturer's protocol (Pierce, USA). Rabbits were immunized by Animal Services of Murex Biotech (University of Cambridge, UK) according to the standard schedule (Harlow & Lane, 1988). Test bleeds were assayed against H1 or H2 proteins either by enzyme-linked immunosorbent assay (ELISA) or dot blots.

Purification of antibodies and Fab fragments

Rabbit polyclonal antibodies were affinity-purified (Harlow & Lane, 1988) using H1 or H2 linked to CNBr-activated Sepharose-4B (Amersham Pharmacia Biotech). The gels in PBS (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 2.7 mM KCl) were packed into 5 ml BioRad Dispo-columns. Filtered antiserum, 20 ml mixed with 80 ml of PBS, was loaded and antibodies were eluted in 10 ml of 0.15 M glycine (pH 2.5) and then 10 ml of 100 mM triethylamine (pH 11.5). Each 600 µl fraction was neutralized with 100 µl of 1 M Tris-HCl (pH 7.4). Second antibodies used in ELISA or blot assays were alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Sigma) diluted 1:2000. IgG was digested into Fab fragments using the ImmunoPure Fab Preparation Kit (Pierce). The secondary antibody was HRP-conjugated anti-Fab (Jackson Immuno Research Lab Inc), diluted 1:2000. Antibody IgG and Fab concentrations were determined with values of E1 % (279 nm) = 14.0 and 14.8, respectively (Nisonoff *et al*., 1970).

Control antibodies

CDHC was detected using goat polyclonals C-18 and S-19 (Santa Cruz Biotechnology), to the C and N termini (working dilution 1:100). Anti-goat IgG (Sigma) was diluted 1:4000. Rabbit polyclonal IgG,R-325, against residues 4320-4644 of CDHC (working dilution 1:200), was also from Santa Cruz. Mouse monoclonal anti-P150 and anti-P50 (AbCam), against dynactin polypeptides P150 and P50 (working dilutions 1:500); IC74.1 (Covance) to CDIC (working dilution 1:2000); and anti-MAP2 (Sigma) (working dilution 1:500) were all detected with AP or HRP-conjugated anti-mouse IgG (Sigma) against mouse Fc (working dilution 1:2000).

Microtubule and dynein preparation

Tubulin was purified from pig brain and microtubules were polymerized in BRB80 (80 mM Pipes (pH 6.8), 1 mM EGTA, 2 mM $MgSO_4$, 1 mM DTT, 0.1 mM PMSF) containing 20 µM Taxol (Hyman *et al*., 1991). Pig brain dynein was prepared using a combination of published methods (Amos, 1989; Paschal *et al*., 1987). N-terminal protein sequencing was carried out by Sew Peak-Chew (IRC, MRC centre).

In vitro motility assay

Motility was observed by video-enhanced differential interference contrast microscopy in a ~10 µl perfusion chamber (Allan, 1993) made from a microscope slide (Chance Propper Ltd., UK) and glass coverslip (No.1, 18 mm × 18 mm, Menzel-Glasser, Fisher, Germany). Dynein (10 µl of 0.1-0.2 mg/ml) was dropped onto the coverslip (drop diameter ~5 mm) and incubated for five minutes (Paschal *et al*., 1987). The glass surfaces were blocked with 10µl of 0.025 % (w/v) BSA (Sigma) in motility buffer (50 mM Tris-HCl (pH 7.4), 50 mM KCl, 0.5 mM EDTA, 5 mM $MgSO_4$) plus 0.1 mM Mg-ATP (Sigma), 20 µM Taxol and Protease Inhibitor Cocktail (5µl per 1 ml) (Sigma) at room temperature for one minute. The chamber was then loaded with 10 µl of a 1:1 mixture of PBS and motility buffer (control), or the same buffer mix containing 1 µg of anti-DHC antibodies, and incubated at 30 °C for three minutes. Free protein was removed by a quick wash with 10 µl of motility buffer and finally 10 µl of 0.1 mg/ml Taxol-stabilised microtubules in motility buffer plus 1-2 mM Mg-ATP were loaded. Captured video frames at one second intervals were analysed using RETRAC (Dr N. Carter, E-mail: ncarter@mcri.ac.uk).

Further processing of digital images was carried out using Photoshop and Illustrator (Adobe). Graphs were plotted using Cricketgraph and Illustrator.

Electron microscopy

The cytoplasmic dynein fraction was diluted into HB buffer (50 mM Pipes-K and 30 mM Hepes-K (pH 7.0), 1 mM EGTA, 2 mM $MgSO_4$, 1 mM DTT, 0.1 mM

Mg-ATP and 0.1 mM PMSF) before negative staining with tannic acid/phosphotungstic acid as described by Kensler *et al*. (1985) or Lanzavecchia & Bellon (1994).

Note added in proof

Mocz & Gibbons have recently modelled a detailed atomic structure for a ring of six AAA modules in a dynein motor domain (Mocz, G. & Gibbons, I. R. (2001). *Structure*, **9**, 93-103).

Acknowledgements

We thank the Kazusa DNA Research Institute, Japan for providing cDNA clone KIAA0325. We are grateful to Ross Jakes, David Owen, Brian Pope and Teresa Langford for technical advice at various stages in making peptides and antibodies, Brad Amos for help with light microscopy, Jan Löwe, John Kendrick-Jones and Tony Crowther for additional advice.

References

Allan, V. (1993). Assay of membrane motility in interphase and metaphase *Xenopus* extracts. In *Methods in Cell Biology* (Scholey, J. M., ed.), vol 39, pp. 203-226, Academic Press, San Diego.

Amos, L. A. (1989). Brain dynein crossbridges microtubules into bundles. *J. Cell Sci.* **93**, 19-28.

Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D. & Huber, R. (2000). The structures of HsIU and the ATP-dependent protease HsIU-HsIV. *Nature,* **403**, 800-805.

Burgess., A. S. (1995). Rigor and relaxed outer dynein arms in replicas of cryofixed motile flagella. *J. Mol. Biol.* **250**, 52-63.

Criswell, P. S., Ostrowski, L. E. & Asai, D. J. (1996). A novel cytoplasmic dynein heavy chain: expression of DHC1β in mammalian ciliated epithelial cells. *J. Cell Sci.* **109**, 1891-1898.

Gee, M. & Vallee, R. (1998). The role of the dynein stalk in cytoplasmic and flagellar motility. *Eur. Biophys. J.* **27**, 466-473.

Gee, M. A., Heuser, J. E. & Vallee, R. B. (1997). An extended microtubule-binding structure within the dynein motor domain. *Nature,* **390**, 636-639.

Gill, S. R., Schroer, T. A., Szilak, I., Steuer, E. R. & Sheetz, M. P. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* **115**, 1639-1650.

Gönczy, P., Pichler, S., Kirkham, M. & Hyman, A. A. (1999). Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage *Caenorhabditis elegans* embryo. *J. Cell Biol.* **147**, 135-150.

Goodenough, U. W. & Heuser, J. E. (1989). Structure of soluble and *in situ* ciliary dyneins visualized by quick-freeze deep-etch microscopy. In *Cell Movement: The Dynein ATPases* (Warner, F. D., Satir, P. & Gibbons, I. R., eds), vol 1, pp. 121-140, Alan Liss Inc., New York.

Habura, A., Tikhonenko, I., Chisholm, R. L. & Koonce, M. P. (1999). Interaction mapping of a dynein heavy chain. Identification of dimerization and intermediate-chain binding domains. *J. Biol. Chem.* **274**, 15447-15453.

Harlow, E. & Lane, D. (1988). *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Holzbaur, E. L. F. & Vallee, R. B. (1994). Dynein: molecular structure and cellular function. *Annu. Rev. Cell Biol.* **5**, 339-372.

Hopp, T. P. & Woods, K. R. (1981). Prediction of protein antigenic determinations from amino acid sequences. *Proc. Natl Acad. Sci. USA,* **78**, 3824-3828.

Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L. & Mitchison, T. (1991). Preparation of modified tubulins. *Methods Enzymol.* **196**, 478-479.

Kandl, K. A., Forney, J. D. & Asai, D. J. (1995). The dynein genes of *paramecium tetraurelia*: the structure and expression of the ciliary β and cytoplasmic heavy chains. *Mol. Biol. Cell,* **6**, 1549-1562.

Karki, S. & Holzbaur, E. L. F. (1999). Cytoplasmic dynein and dynactin in cell division and intracellular transport. *Curr. Opin. Cell Biol.* **11**, 45-53.

Kensler, R. W., Levine, R. J. C. & Stewart, M. (1985). Electron microscopic and optical diffraction analysis of the structure of scorpion muscle thick filaments. *J. Cell Biol.* **101**, 395-401.

King, S. M. (2000). AAA domains and organization of the dynein motor unit. *J. Cell Sci.* **13**, 2521-2526.

Kitagawa, T. A. T. (1976). Enzyme coupled immunoassay of insulin using a novel coupling reagent. *J. Biochem.* **79**, 233-236.

Koonce, M. P. (1997). Identification of a microtubule-binding domain in a cytoplasmic dynein heavy chain. *J. Biol. Chem.* **272**, 19714-19718.

Koonce, M. & Samso, M. (1996). Overexpression of cytoplasmic dynein's globular head causes a collapse of the interphase microtubule network in *Dictyostelium*. *Mol. Biol. Cell,* **7**, 935-948.

Koonce, M. P. & Tikhonenko, I. (2000). Functional elements within the dynein microtubule-binding domain. *Mol. Biol. Cell,* **11**, 523-529.

Langer, T. (2000). AAA proteases: cellular machines for degrading membrane proteins. *Trends Biochem. Sci.* **25**, 247-251.

Lanzavecchia, S. & Bellon, P. L. (1994). Three-dimentional reconstructions of accessory tubules observed in the sperm axonemes of two insect species. *J. Struct. Biol.* **113**, 225-237.

Lee-Eiford, A., Ow, R. A. & Gibbons, I. R. (1986). Specific cleavage of dynein heavy chains by ultraviolet irradiation in the presence of ATP and vanadate. *J. Biol. Chem.* **261**, 2337-2342.

Li, M. G., McGrail, M., Serr, M. & Hays, T. S. (1994). *Drosophila* cytoplasmic dynein, a microtubule motor that is asymmetrically localized in the oocyte. *J. Cell Biol.* **126**, 1475-1494.

Liu, F. T., Zinnecker, M., Hamaoka, T. & Katz, D. H. (1979). New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry,* **18**, 690-693.

Lyadurai, S. J., Li, M. G., Gilbert, S. P. & Hays, T. S. (1999). Evidence for cooperative interactions between the two motor domains of cytoplasmic dynein. *Curr. Biol.* **9**, 771-774.

Mazumdar, M., Mikami, A., Gee, M. A. & Vallee, R. B. (1996). *In vitro* motility from recombinant dynein

heavy chain. *Proc. Natl Acad. Sci. USA,* **93**, 6552-6556.

Mikami, A., Paschal, B. M., Mazumdar, M. & Vallee, R. B. (1993). Molecular cloning of the retrograde transport motor cytoplasmic dynein (MAP1c). *Neuron,* **10**, 787-796.

Mocz, G., Helms, M. K., Jameson, D. M. & Gibbons, I. R. (1998). Probing the nucleotide binding sites of axonemal dynein with the fluorescent nucleotide analogue 2′(3′)-*O*-(*N*-methylanthraniloyl)-adenosine 5′-triphosphate. *Biochemistry,* **37**, 9862-9869.

Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. (1999). AAA: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9**, 27-43.

Nisonoff, A., Macdonald, A. B., Hopper, J. E. & Daugharty, H. (1970). Quantitative studies of idiotypic antibodies. *Fed. Proc. Fed. Am. Soc. Expt. Biol.* **29**, 72-77.

Paschal, B. M., Shpetner, H. S. & Vallee, R. B. (1987). MAP1C is a microtubule-activated ATPase which translocates microtubules *in vitro* and has dynein-like properties. *J. Cell Biol.* **105**, 1273-1282.

Paschal, B. M., Shpetner, H. S. & Vallee, R. B. (1993). Purification of brain cytoplasmic dynein and characterization of its *in vitro* properties. In *Methods in Cell Biology* (Scholey, J. M., ed.), vol 39 edit., pp. 181-191, Academic Press, San Diego.

Patel, S. & Latterich, M. (1998). The AAA team: related ATPases with diverse functions. *Trends Cell Biol.* **8**, 65-71.

Rost, B. & Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584-599.

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

Samso, M., Radermacher, M., Frank, J. & Koonce, M. P. (1998). Structural characterization of a dynein motor domain. *J. Mol. Biol.* **276**, 927-937.

Schroer, T. A. (1994). Structure, function and regulation of cytoplasmic dynein. *Curr. Biol.* **6**, 69-73.

Schroer, T. A. & Sheetz, M. P. (1991). Two activators of microtubule-based vesicle transport. *J. Cell Biol.* **115**, 1309-1318.

Vaisberg, E. A., Koonce, M. P. & McIntosh, J. R. (1993). Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *J. Cell Biol.* **123**, 849-858.

Vaisberg, E. A., Grissom, P. M. & McIntosh, J. R. (1996). Mammalian cells express 3 distinct dynein heavy-chains that are localized to different cytoplasmic organelles. *J. Cell Biol.* **133**, 831-842.

Vale, R. D. (2000). AAA proteins: Lords of the ring. *J. Cell Biol.* **150**, F13-F19.

Vallee, R. (1993). Molecular analysis of the microtubule motor dynein. *J. Biol. Chem.* **90**, 8769-8772.

Vallee, R. B. & Sheetz, M. P. (1996). Targeting of motor proteins. *Science,* **271**, 1539-1544.

Wilkinson, D. L. & Harrison, R. G. (1991). Predicting the solubility of recombinant proteins in *Escherichia coli*. *Biotechnology,* **9**, 443-448.

Zhang, Z. Z., Tanaka, Y., Nonaka, S., Aizawa, H., Kawasaki, H., Nakata, T. & Hirokawa, N. (1993). The primary structure of rat-brain (cytoplasmic) dynein heavy-chain, a cytoplasmic motor enzyme. *Proc. Natl Acad. Sci. USA,* **90**, 7928-7932.

Edited by M. F. Moody

(Received 14 December 2000; received in revised form 20 February 2001; accepted 22 February 2001)