Comparison of the Motile and Enzymatic Properties of Two Microtubule Minus-End-Directed Motors, ncd and Cytoplasmic Dynein†

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ABSTRACT: Cytoplasmic dynein and ncd, a kinesin-related protein from *Drosophila*, are motor proteins that move toward the minus ends of microtubules, while kinesin moves to the microtubule plus end. In previous work, we examined the nucleotide dependence of motility and enzymatic activity by kinesin [Shimizu, T., Furusawa, K., Ohashi, S., Toyoshima, Y. Y., Okuno, M., Malik, F., & Vale, R. D., (1991) *J. Cell Biol. 112*, 1189−1197]. In this study, we examined these activities of the cytoplasmic dynein from bovine brain and ncd in order to explore what enzymatic features might be shared by these two minus-end-directed motors. Both ncd and cytoplasmic dynein demonstrated an activation of ATPase activity upon the addition of microtubules (30-fold and 6-fold, respectively). A significant difference between ncd and cytoplasmic dynein was their relative sensitivity to vanadate and to aluminum fluoride. In contrast to cytoplasmic dynein, ncd polypeptide was not cleaved by UV−vanadate treatment, and its ATPase and motility were unaffected by vanadate (up to 0.1 mM). When the nucleotide requirement for movement as examined using a battery of 20 nucleotides and nucleotide analogues, cytoplasmic dynein was found to exhibit a specificity very similar to that of axonemal dyneins from *Tetrahymena*. Surprisingly, however, the nucleotide specificities of in vitro motility produced by ncd or its construct, GST/MC1 (a fusion protein of glutathione *S*-transferase and 210−700 of the predicted ncd amino acid sequence), were quite distinct from that of kinesin. Thus, the nucleotide specificity profiles of members of the kinesin motor superfamily do not appear to be identical.

A variety of intracellular movements, such as ciliary beating, organelle transport, and chromosome movements, depend upon intracellular microtubules. Microtubule-based motility is driven by two distinct mechanochemical enzymes, dynein and kinesin, that are unrelated in their amino acid sequences.

Dynein was originally found as a ciliary or flagellar axonemal ATPase (Gibbons, 1963), which was later shown to be responsible for producing sliding between adjacent outer doublet microtubules (Summers & Gibbons, 1971; Shingyoji et al., 1977). More recently, a cytoplasmic form of dynein has been found in various types of cells (Porter & Johnson, 1989; Paschal et al., 1987), where it is considered to transport organella and chromosomes (Schroer et al., 1989; Steuer et al., 1990; Pfarr et al., 1990). Both axonemal and cytoplasmic dyneins are very large protein complexes with two or three globular domains (heads) connected by short rods (stems) to a common base (Johnson & Wall, 1983; Vallee et al., 1988). The globular heads are composed primarily of a very large heavy chain (500 kDa molecular mass), whose primary structure has recently been deduced from its cDNA sequence (Ogawa, 1991; Gibbons et al., 1991; Koonce et al., 1992). A remarkable feature of the sequence is the presence of four consensus sites for ATP binding. In addition to the heavy chains, dynein contains intermediate and light chains, some of which have been localized to the base of molecule (King & Witman, 1990). All dyneins examined move unidirectionally on microtubules toward their minus (less dynamic) ends (Vale & Toyoshima, 1988).

Kinesin was found in squid axoplasm as a microtubule plus-end-directed motor (Vale et al., 1985), but now it is known to exist in non-neuronal cells as well. The molecule is a tetramer of two 120 kDa heavy chains and two 70 kDa light chains (Kuznetsov & Gelfand, 1986; Bloom et al., 1988). The N-terminal 400 amino acid residues of its heavy chain form a globular motor domain containing a single ATP binding site, and the rest forms a large α-helical coiled coil and a small globular tail domain. The light chain associates with the C-terminal region of the heavy chain. Morphologically, kinesin appears by electron microscopy as an elongated molecule with two globular domains at each end separated by a long rod (Hisanaga et al., 1989). This overall shape is similar to that of myosin, but is quite distinct from that of dynein.

Recent genetic analyses have uncovered numerous genes that have domains whose predicted amino acid sequences bear homology to the kinesin motor domain (Vale & Goldstein, 1990; Endow & Titus, 1992). ncd, the product

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of the nonclaret disjunctional gene (*ncd*), which functions in chromosome segregation during female meiosis of *Drosophila*, is a well-studied example of a kinesin-related protein. However, in contrast to kinesin, ncd's motor domain is at the C-terminus of the 80 kDa polypeptide. The ncd polypeptide expressed in bacteria dimerizes (Chandra et al., 1993) and has motor activity as determined by an in vitro motility assay. Surprisingly, however, it is a minus-end-directed microtubule motor, which is opposite to kinesin but the same direction as produced by dynein (Walker et al., 1990; McDonald et al., 1990). This polarity recently was shown to originate from the motor domain, since neither removal of the kinesin or ncd rod domain nor attachment of the kinesin rod domain to the N-terminus of kinesin motor affects the polarity of movement (Stewart et al., 1993).

The finding that cytoplasmic dynein and ncd, two structurally different motors, move in the same direction along a microtubule raises the intriguing question of how the direction of movement of a motor is specified. As a beginning to explore this problem, it is first necessary to characterize the motile and enzymatic properties of the cytoplasmic dynein, ncd, and kinesin motors. In this study, we wished to compare side-by-side the properties of the two minus-end-directed motors, cytoplasmic dynein and ncd. We found that the vanadate sensitivity of ncd is more akin to that of kinesin than that of cytoplasmic dynein. However, the nucleotide specificity profile of the motility of ncd was different from that of kinesin or dynein. This finding suggests that members of the kinesin superfamily have distinct interaction properties with nucleotides.

MATERIALS AND METHODS

Materials. Taxol was a generous gift from Dr. M. Suffness of the National Cancer Institute (NIH, Bethesda, MD). AMPPNP[1] was from Sigma (St. Louis, MO). ATP and the analogues used herein were the same as those described previously (Shimizu et al., 1991).

Tubulin free from microtubule-associated proteins (MAPs) was prepared from bovine brain high-speed supernatant, by using cycles of polymerization−depolymerization and DEAE-Sephacel column chromatography, as described by Murphy and Borisy (1975).

ncd, the product of the *ncd* gene but with truncation of several amino acid residues at the N-terminus, as described by McDonald et al. (1990), was expressed in *Escherichia coli* and partially purified by Pipes precipitation, as reported by the same authors. ncd was frozen in aliquots with 0.3 M NaCl in liquid nitrogen and stored at −80 °C.

GST/MC1 (Chandra et al., 1993), a fusion protein of glutathione *S*-transferase and residues 210−700 of the predicted ncd protein, was expressed in *E. coli* from the DNA fragment, pGEX-N210, and partially purified by S-Sepharose column chromatography. The eluent at 0.2 M NaCl was used for further analysis. pGEX-N210, encoding amino acids 210−700 (N210) of ncd, was prepared by polymerase chain reaction (PCR) from a cDNA clone of ncd (a gift of Prof. L. S. B. Goldstein, University of California−San Diego). The PCR primers were 5′-GGC CCG GGT AAG ACA AAG TAC GAA AAG CA-3′ and 5′-GGG AAT TCT TAT TTA TCG AAA CTG CCG C-3′. *Pfu* DNA polymerase (Stratagene) was used in the reaction. The resulting PCR products were digested with *Sma*I and *Eco*RI and ligated into pGEX-2T digested with these enzymes. No alteration in the inserts was confirmed by DNA sequence analysis using a T7 sequencing kit (Pharmacia P-L Biochemical).

Cytoplasmic dynein was obtained from brains according to a modification of kinesin preparation of F. Malik and R. D. Vale (unpublished), which is briefly summarized here. High-speed supernatant (100000*g* for 45 min) of bovine brain homogenate was warmed in the presence of 33% glycerol to polymerize microtubules, and then apyrase was added to deplete ATP, ADP, and other nucleoside polyphosphates. The microtubules were then collected by centrifugation at 100000*g* for 1 h and extracted sequentially by incubation with 0.1 M Pipes−NaOH (pH 6.8), 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and (1) 2mM AMPPNP, (2) 2 mM GTP, and (3) 2 mM ATP in the presence of taxol. The ATP extract was applied to a 2 mL phosphocellulose column, and cytoplasmic dynein was eluted with 0.2 M NaCl in the preceding buffer. Cytoplasmic dynein was further purified by centrifugation through a sucrose gradient (5−20%) in 50 mM Mes−NaOH (pH 6.8) containing 2 mM $MgCl_2$, 0.5 mM EDTA, and DTT for 10 h at 38 000 rpm and 5 °C using an RPS40T rotor on a Hitachi 70P ultracentrifuge. Fractions were checked by SDS−PAGE for cytoplasmic dynein.

Kinesin was prepared from squid optic lobes by the method of Vale et al. (1985).

Methods. Protein concentrations were determined by the method of Lowry et al. (1951) or by a modified Bradford method (Read & Northcote, 1981), using bovine serum albumin as a standard.

Microtubule translocation in vitro was assayed as described (Vale & Toyoshima, 1989; Shimizu et al., 1991) at 22−24 °C for ncd and 25 °C for GST/MC1, squid kinesin, and cytoplasmic dynein motility. The assay conditions are described in Table 2.

The microtubule translocation assay of GST/MC1 is described here. The construct protein at 0.1 mg/mL, with 0.2 mg/mL casein in a solution of 10 mM phosphate (pH 7.0), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and DTT, was applied to the glass surface and then washed with the assay buffer plus 1 mM nucleotide, and then microtubules in the assay buffer plus 1 mM nucleotide were perfused. In Pipes buffer in the presence of 0.1 M NaCl, as the assay solution that was used for ncd as before, however, microtubules were translocated on the surface coated with GST/MC1 for 20−30 s, but they easily diffused away, probably due to weak binding of the construct molecules to the glass surface in the presence of 0.1 M NaCl. When the salt concentration was reduced to 0.05 M, the translocation was observed for more than 10 min. It should be noted that,

[1] Abbreviations: AMPPNP, β,γ-imidoadenosine 5′-triphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); dideoxy-ATP, 2′,3′-dideoxyadenosine 5′-triphosphate; dimethyl-ATP, N^6,N^6-dimethyladenosine 5′-triphosphate; 8-azido-ATP; 8-azidoadenosine 5′-triphosphate; 8-bromo-ATP, 8-bromoadenosine 5′-triphosphate; EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; etheno-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; FTP, formycin 5′-triphosphate; MAP(s), microtubule-associated protein(s); monomethyl-ATP, N^6-methyladenosine 5′-triphosphate; Mops, 3-morpholinopropanesulfonic acid; NTP, nucleoside 5′-triphosphate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PRTP, purine riboside 5′-triphosphate; 2′-dATP, 2′-deoxyadenosine 5′triphosphate; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

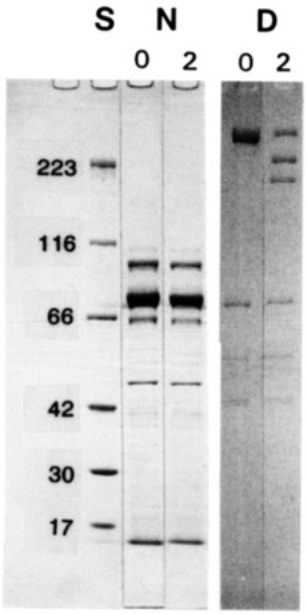

FIGURE 1: UV−vanadate treatment of ncd fraction and cytoplasmic dynein. ncd fraction and cytoplasmic dynein were subjected to near-UV irradiation with a thin-layer-chromatography illuminator in the presence of 2 mM ATP and 0.1 mM vanadate on ice for 2 h. SDS−PAGE was carried out on 4−20% gradient gels. The heavy chain of cytoplasmic dynein was cleaved, whereas the pattern of ncd fraction was not affected. N and D indicate ncd fraction and cytoplasmic dynein, and 0 and 2 are the irradiation period in hours. Molecular markers are as follows: 223 kDa, myosin heavy chain; 116 kDa, β-galactosidase; 66 kDa, bovine serum albumin; 42 kDa, aldolase; 30 kDa, carbonic anhydrase; 17 kDa, myoglobin.

although Chandra et al. (1993) described that GST/MC1 eluted from S-Sepharose exhibited poor motility, we observed microtubule translocation consistently with our GST/MC1 eluted from S-Sepharose with a buffer solution containing 0.2 M salt. We do not know the reason for this difference.

The ATP or NTP turnover activity of cytoplasmic dynein was measured in an assay mixture consisting of 50 mM Mops−NaOH (pH 7.0), 2 mM $MgCl_2$, 1 mM NTP and 0.02−0.1 mg/mL enzyme at 25 °C. For the enzyme activity of the ncd fraction, the reaction mixture contained 80 mM Mops−NaOH (pH 7.0), 2 mM $MgCl_2$, 1 mM EGTA, 0.1 M NaCl, 1 mM NTP, and 0.02−0.2 mg/mL protein, unless otherwise described. The reaction was terminated by adding final 0.3 M perchloric acid, and the phosphate liberated was determined by the modified malachite green method (Shimizu & Furusawa, 1986). For the microtubule activation of cytoplasmic dynein or ncd, microtubules polymerized by taxol were pelleted by centrifugation and resuspended in a GTP-free buffer solution to remove inherent GTP.

The polyacrylamide gel electrophoresis in the presence of SDS (SDS−PAGE) was carried out on 4−20% gradient gels from Daiichi Pure Chemicals (Tokyo, Japan).

RESULTS

UV−Vanadate Photocleavage Reaction of Cytoplasmic Dynein and ncd. A characteristic property of the dynein family is the cleavage of the heavy chain by near-UV irradiation in the presence of ATP and vanadate (Lee-Eiford et al., 1986). We wished to examine whether ncd would undergo a similar photocleavage reaction.

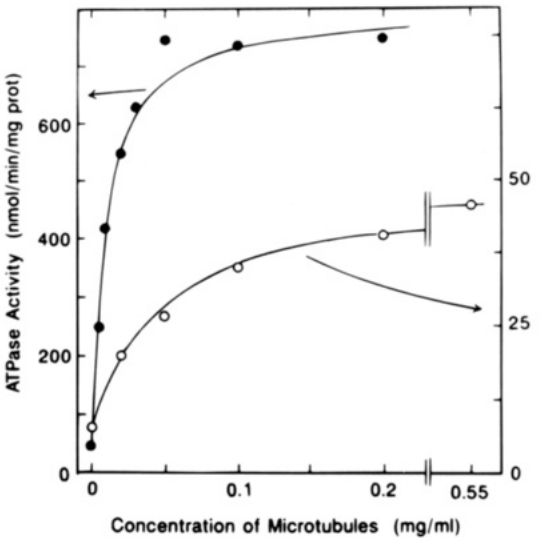

FIGURE 2: Microtubule stimulation of the ATPase activities of ncd fraction and cytoplasmic dynein. The ATPase assay was performed as described in Materials and Methods at 25 °C. The assay mixture contained 50 mM Mops−NaOH (pH 7.0), 2 mM $MgCl_2$, 0.5 mM ATP, and 0.05 mg/mL cytoplasmic dynein (○) or 0.1 M Mes−NaOH (pH 6.8), 12 mM NaCl, 2 mM $MgCl_2$, 0.5 mM ATP, and 0.02 mg/mL ncd fraction (●). When microtubules were included, the ATP turnover by microtubules only was measured for the determination of the activity of the motor enzymes. The solid curves represent the calculated hyperbolas; for ncd, the maximum is 800 nmol/min/mg of protein with half-maximal stimulation given at 0.01 mg of microtubules/mL, and for cytoplasmic dynein, it is 48 nmol/min/mg of protein with half-maximal stimulation given at 0.05 mg of microtubules/mL. Note that the abscissa scales are different for the two enzymes.

Figure 1 shows that the heavy chain of cytoplasmic dynein was cleaved by UV−vanadate treatment, with no sign of heterogeneous cleavage [Neely et al., 1990; see Koonce et al. (1992)]. On the other hand, the 73 kDa ncd polypeptide was unaffected under the same experimental conditions. The heavy and light chains of kinesin were shown to be insensitive to this treatment (Lye et al., 1989). Thus, the UV−vanadate photocleavage appears to distinguish between dynein and ncd motors.

Enzymological Characteristics. Both ncd and cytoplasmic dynein were previously shown to be microtubule-stimulated ATPases (Chandra et al., 1993; Shpetner et al., 1988). Here we performed a side-by-side comparison of the microtubule stimulation of the two motor ATPases (Figure 2). The ATP turnover in the absence of microtubules was low for both motor enzymes, in the ranges of 20−30 and 5−10 nmol/min/mg for ncd and cytoplasmic dynein, respectively, at 25 °C. In the presence of microtubules, ncd ATPase activity was stimulated by ca. 30-fold, which was much higher than the stimulation of cytoplasmic dynein (6-fold). The microtubule concentration needed for half-maximal stimulation was also lower for ncd (0.01 mg of microtubules/mL) compared to cytoplasmic dynein (0.05 mg of microtubules/mL).

Vanadate, a potent inhibitor of dynein motile and enzymatic (Paschal et al., 1987), did not affect the basal rate of ATP turnover by ncd (data not shown). Chandra et al. (1993) reported that microtubule-stimulated ATPase of ncd was not influenced by vanadate. Kinesin is reported to be inhibited by vanadate but is relatively insensitive (Cohn et al., 1989). Aluminum fluoride (AlF_4), a substance that inhibits many phosphate-metabolizing enzymes [Maruta et al., (1993) and references cited therein], was also shown to be a fairly

Table 1: AlF_4 Inhibition of Microtubule-Activated ATPases of ncd and Cytoplasmic Dynein[a]

$AlCl_3$ concentration (mM)	ATPase activity (nmol/min/mg of enzyme)	
	ncd	cytoplasmic dynein
0	506	62.4
0.001	462	55.7
0.01	450	37.7
0.1	296	7.8
1.0	254	nd

[a] The ATPase activities of ncd and cytoplasmic dynein were assayed in the mixture containing 20 mM Mops−NaOH (pH 7.0), 50 mM NaCl, 2 mM $MgCl_2$, 0.5 mM ATP, 5 mM NaF, 0.03 mg of enzyme/mL, and 0.55 or 0.3 mg of microtubules/mL for cytoplasmic dynein and ncd, respectively, and various concentrations of $AlCl_3$ as indicated in the Table. AlF_4 forms spontaneously upon mixing Al^{3+} and F^-. The time course of phosphate production at 25 °C was determined by the malachite green assay. Microtubules were used after polymerization from tubulin by taxol and after pelleting and resuspension in GTP-free fresh buffer solution and showed negligible ATPase activity. nd means not detectable.

effective inhibitor of the cytoplasmic dynein ATPase (Table 1), while its effect was less potent on the ncd ATPase. Ciliary dynein from *Tetrahymena* was as sensitive as its cytoplasmic counterpart, while kinesin was affected in the same manner as ncd (T. Shimizu, unpublished). Thus, sensitivity to vanadate and AlF_4 may constitute an important difference between the dynein and kinesin superfamily motors.

We examined the ATPase activities of ncd and cytoplasmic dynein under various pH's and ionic strength conditions (Figure 3). The pH dependencies of microtubule-stimulated ATPases of ncd and cytoplasmic dynein were similar to each other, showing highest activity at neutral pH. ATPase stimulation was high at lower ionic strengths for both motor enzymes, but ncd exhibited a peak between 0.15 and 0.2 M NaCl.

Motile Characteristics. Microtubule translocation on an ncd-coated glass surface was examined in 80 mM Pipes (pH 6.8), 1 mM $MgCl_2$, and 1 mM EGTA containing 0.1 M NaCl (McDonald et al., 1990). Application of a fairly high concentration of the protein (ca. 0.5 mg/mL) to the flow chamber was necessary for successful microtubule motility, perhaps because the ncd preparation was only partially pure. At a protein concentration of 0.1 mg/mL, the motility was not consistently observed, while at 1 mg/mL, large aggregates and bundles of microtubules were formed that displayed poor motility. Under optimal conditions, the speed at 1 mM ATP was 0.14−0.18 μm/s, consistent with the value reported by McDonald et al. (1990). In the absence of added NaCl (0.1 M), the motility was still observed, but the speed at 1 mM ATP was slower (0.05−0.08 μm/s) and fewer microtubules were moving while most microtubules were stuck on the surface.

We also examined the motility produced by GST/MC1, a GST fusion construct of ncd devoid of the N-terminal region, that has the second, ATP-independent microtubule binding site (Chandra et al., 1993). This protein exhibited microtubule motility at 0.14−0.22 μm/s at 1 mM ATP under the experimental conditions described in Materials and Methods.

Cytoplasmic dynein from brain adsorbed onto a glass coverslip surface translocated microtubules in the presence of 1 mM ATP at a speed of ca. 1 μm/s, similar to the values of Paschal et al. (1987). The optimal concentration for movement of cytoplasmic dynein applied to a glass surface was 0.05−0.2 mg/mL. Beyond these values, the translocation was less smooth.

Microtubule motility produced by ncd was reported to be sensitive to vanadate (Walker et al., 1990), but we found that vanadate up to 0.1 mM did not affect the motility speed with 1 mM ATP at all. This result is consistent with the insensitivity of the ATPase activity of the ncd fraction (Chandra et al., 1993; this work, see above) and with the insensitivity of ncd to UV−vanadate treatment. The reason for the discrepancy in vanadate sensitivity may be because Walker et al. (1990) used a bacterial cell lysate supernatant for the motility assay, which might have been more sensitive to the change in assay conditions, whereas we used a partially purified ncd preparation.

When the ATP concentration dependence of cytoplasmic dynein and GST/MC1 motor activity was analyzed by the Eadie−Hofstee plot, a straight line was obtained with GST/MC1, whereas the plot had a bend with cytoplasmic dynein (Figure 4). The apparent K_m and V_{max} values for GST/MC1 were 0.10 mM and 0.20 μm/s, and those for cytoplasmic dynein were 0.033 and 0.28 mM and 0.71 and 1.24 μm/s, respectively. The reason for the bend in this plot for cytoplasmic dynein is not known, but since the heavy chains with the putative ATPase sites are thought to be the same (Neely et al., 1990; Koonce et al., 1992), this might indicate negative cooperativity between the ATPase sites or some function of a regulatory ATP binding site(s).

Substrate Specificity of Squid Kinesin, ncd, GST/MC1, and Cytoplasmic Dynein Motility. We previously tested the substrate specificities of in vitro motility by axonemal dynein and brain kinesin using 15 ATP analogues and found that kinesin had a very broad specificity compared with axonemal dyneins. Here, we tested the nucleotide specificity of squid optic lobe kinesin in the presence of 0.1 M NaCl (Table 2), which was used for assaying ncd motility (see the following). Although the translocation of microtubules by kinesin in the presence of 0.1 M NaCl was slower (0.2 μm/s) than in its absence (0.76 μm/s), kinesin again exhibited broad substrate specificity. Table 2 also shows the substrate specificity of cytoplasmic dynein at 1 mM NTP, which was similar to that of other dyneins (Shimizu et al., 1991); only deoxy-ATPs, monomethyl-ATP, and (S_P)-ATPαS supported the translocation. Thus, all of the dyneins investigated so far are similar to each other in terms of substrate specificity.

Unexpectedly, the substrate specificity of ncd motility was similar to that of dynein (Table 2) and very different from that of kinesin. Besides ATP, which was the best substrate, only deoxy-ATPs and monomethyl-ATP supported the motility. Furthermore, GTP, which supports kinesin motility (Table 2), did not support ncd motility [see also Walker et al. (1990)].

In addition to the C-terminal motor domain, ncd also contains an N-terminal nucleotide-independent microtubule binding site. If this site engages with microtubules in the in vitro motility assay, it could impose a load that could impede the movement produced with some nucleotides. To examine this possibility, the nucleotide specificity of GST/MC1, an ncd construct that lacks the N-terminal microtubule binding site, was examined. In addition to the nucleotides

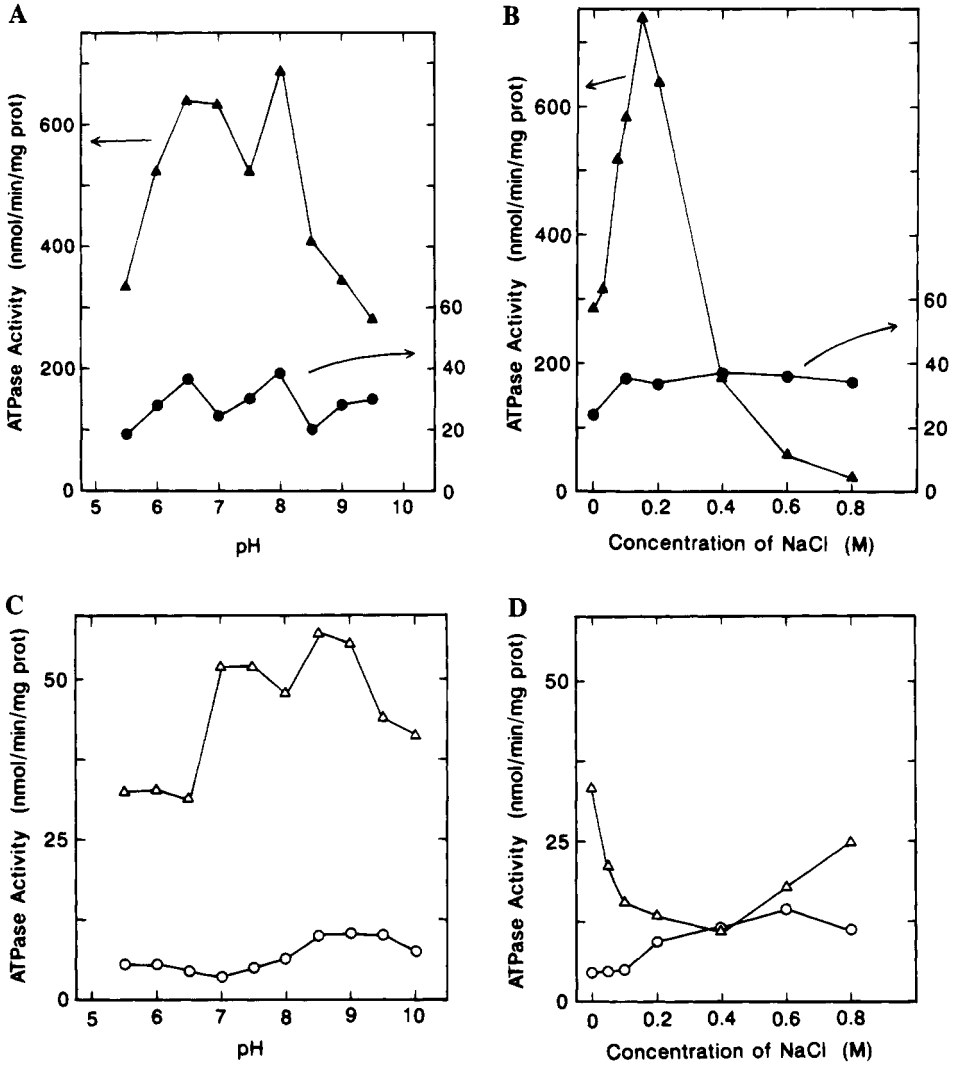

FIGURE 3: pH and NaCl concentration dependence of the ATPase activities of ncd fraction and cytoplasmic dynein. The ATPase assay was performed as described in the legend to Figure 2. For pH dependence, 80 mM Mes−NaOH, Mops−NaOH, and Ches−NaOH were used for pH 5.5−6.5, 7−8, and 8.5−10, respectively. For NaCl concentration dependence, 0.1 M Mes−NaOH (pH 6.8) and 80 mM Mops−NaOH (pH 7.0) were used for ncd fraction and cytoplasmic dynein, respectively. (A and B) ncd fraction: (C and D) cytoplasmic dynein. (A and C) pH dependence; (B and D) NaCl concentration dependence. The ATPase assay was performed in the presence (△, ▲) and absence (○, ●) of 0.5 mg of microtubules/mL. Note that, panels for A and B, the abscissa scales differ according to the presence of microtubules in the assay mixture.

described earlier, dimethyl-ATP, etheno-ATP, (S_P)-ATPαS, GTP, and ITP supported motility by GST/MC1. Nevertheless, the nucleotide specificity profile of GST/MC1 was quite distinct from that of kinesin. For instance, whereas FTP, etheno-ATP, GTP, ITP, and CTP elicited comparable velocities of microtubule translocation by kinesin, only etheno-ATP, GTP, and ITP supported motility by GST/MC1. This difference is not due to the problem of GST/MC1 being at the borderline of observable velocities with these analogues since, at low concentrations of ATP such as 0.01 mM, microtubule translocation at 0.008 μm/s could be detected. Analogues that supported extremely slow kinesin movement (i.e., 8-bromo-ATP, 8-azido-ATP, and PRTP) did not support GST/MC1 motility, although in this case it is possible that the ncd motility was not sufficiently robust to detect such very slow movement. We also note that dimethyl-ATP elicits much slower kinesin movement than ATP, whereas this analogue works almost as well as ATP in the case of GST/MC1. Hence, the ncd motor domain in the GST/MC1 protein has a substrate specificity profile very distinct from those of both kinesin and cytoplasmic dynein.

DISCUSSION

Comparison between ncd and Cytoplasmic Dynein. ncd contains a domain of about 350 amino acid residues that has a high level of amino acid identity (40%) to the motor domain of kinesin, but has no significant amino acid homology to the heavy chain of dynein. The size of the ncd force-generating head is also similar to that of kinesin (Chandra et al., 1993), but is much smaller than that of dynein. On the basis of these structural criteria, one might expect that the motile and enzymatic properties of ncd would be closely related to those of kinesin. The finding that ncd moves in the opposite direction from kinesin on a microtubule, however, indicates that ncd shares at least one characteristic with cytoplasmic dynein. On the other hand, very recently Lockhart and Cross (1994) investigated the kinetic properties of ncd ATPase and found that they are similar to those of kinesin. In this work, we have compared the motility and ATPase activity of these two motors to determine what other features might be shared by these two minus-end-directed motors. It should be noted that ncd in

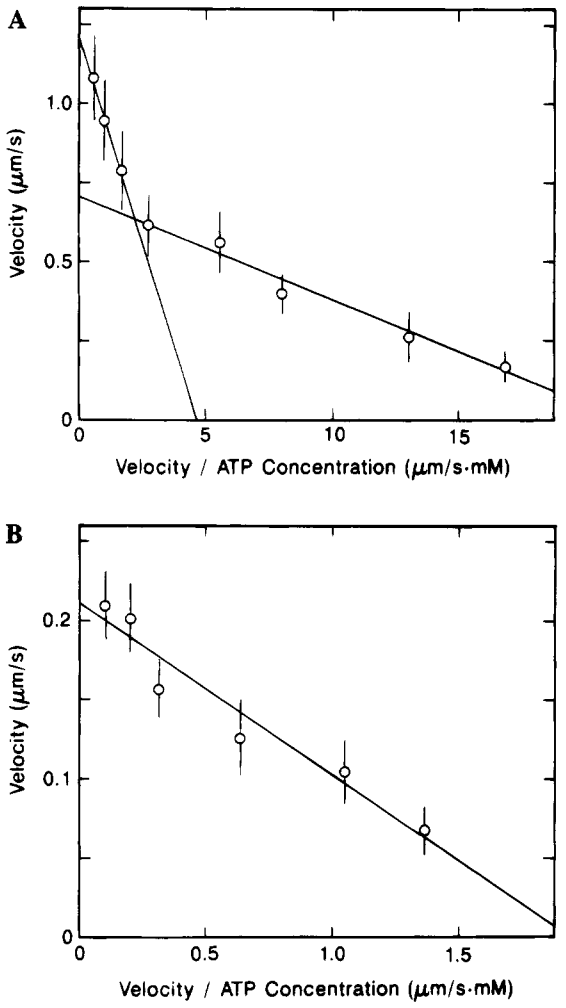

FIGURE 4: Eadie–Hofstee plots of microtubule translocation rates by cytoplasmic dynein and GST/MC1. The microtubule translocation assay was performed as described in the Table 2 with various concentrations of ATP. (A) cytoplasmic dynein; (B) GST/MC1. The plot for cytoplasmic dynein has a bend, and the apparent K_m for ATP and V_{max} for the higher V_{max} are 0.28 mM and 1.24 μm/s and those for the lower V_{max} are 0.033 mM and 0.71 μm/s, respectively. The plot for GST/MC1 was straight, and the apparent K_m and V_{max} values are 0.10 mM and 0.20 μm/s, respectively. The bars represent standard deviations.

vivo may have light or intermediate chains, as kinesin does. It may be very interesting to compare the characteristics of possible holo-ncd to those of bacterially expressed ncd used herein, when the former becomes available in the future.

Dyneins, including cytoplasmic dynein, are potently inhibited by vanadate in both their enzymatic and motile acitivities (Kobayashi et al., 1978; Gibbons et al., 1978; Paschal & Vallee, 1987). Herein we clearly showed that the motility and ATPase activities of ncd were insensitive to vanadate. This is consistent with the fact that the cytoplasmic dynein heavy chain was cleaved by the UV–vanadate treatment, whereas the ncd polypeptide pattern was not affected by the same treatment. Chandra et al. (1993) also showed the insensitivity of ncd's microtubule-stimulated ATPase activity to vanadate. In this regard, ncd is similar to kinesin but not to dynein; the sensitivity of kinesin is reported to depend on the ATP concentration, but it is definitely less than that of dynein (Wagner et al., 1989; Cohn et al., 1989). Another phosphate analogue, aluminum fluoride, was a fairly potent inhibitor of dyneins, but it had much less of an effect on kinesin and ncd ATPases (Table 1). Therefore, vanadate and aluminum fluoride sensitivities mark an important and conspicuous difference between cytoplasmic dynein and ncd.

The enzymatic activities of ncd and cytoplasmic dynein seemed to have some similar properties. The two motors exhibited low ATP turnover activity in the absence of microtubules, but were stimulated by relatively low concentrations of microtubules. The extent of activation, however, was much larger with ncd than with cytoplasmic dynein, which may seem inconsistent with faster translocation by the latter. However, we have already reported that the NTP turnover number does not always correlate with the translocation speed (Shimizu et al., 1991).

Previously, we investigated the nucleotide specificities of motile activities of muscle heavy meromyosin, axonemal dynein, and brain kinesin using a battery of ATP analogues, which indicated that these three classes of motors can be distinguished from one another by their nucleotide fingerprints (Shimizu et al., 1991; see the following). In this study, we examined the specificity of the motile activity of ncd and its shorter construct, GST/MC1. The specificity of GST/MC1 was broader than that of full-length ncd, a difference that may arise from the presence of a second, nucleotide-independent microtubule binding site at the N-terminus of ncd (Chandra et al., 1993). The force produced with nucleotides such as GTP, ITP, and etheno-ATP may not be sufficiently large to overcome the load imposed by the nucleotide-independent binding site in the case of full-length ncd, and we may not have been able to detect apparent movement. In any event, the spectrum of nucleotides that supported GST/MC1 motility was significantly smaller compared to that of kinesin. In addition, some nucleotides such as dimethyl-ATP supported GST/MC1 motility at rates comparable to ATP, but produced a greatly reduced motility rate in the case of kinesin. Together, these findings indicate that two members of the kinesin superfamily of motors, conventional kinesin and ncd, demonstrate distinctly different nucleotide specificities.

Nucleotide Fingerprints. In the study described in the preceding paragraph, we previously suggested that nucleotide (substrate) fingerprinting would be applicable for gaining insights into what types of motors might be driving the movements in various in vitro motility systems. This method has revealed, for example, that the motor responsible for mitotic spindle elongation in diatom cells was kinesin-like (Hogan et al., 1992) and has also been applied to other motility systems as well (Hyman & Mitchison, 1991; Schliwa et al., 1991).

Since only axonemal dynein and brain kinesin were examined as microtubule motors in the initial study, it remained to be determined how these results would pertain to cytoplasmic dynein and other kinesin-like proteins. The present study demonstrates that, although there was some quantitative difference, the specificities of dyneins are very similar. Brush border myosin I, a member of the myosin superfamily, also exhibits specificity in actin filament translocation similar to that of skeletal heavy meromyosin, although the speed was about 100-fold lower (K. Collins, Y. Y. Toyoshima, and T. Shimizu, unpublished). In contrast, ncd, a member of the kinesin superfamily, has a nucleotide specificity that is different from that of kinesin. Regarding this matter, more motors from the kinesin superfamily should

Table 2: Substrate Specificity of Motile Activities of ncd Fraction, GST/MC1, Cytoplasmic Dynein, and Squid Optic Lobe Kinesin[a]

nucleotide	microtubule translocation (μm/s) by			
	cytoplasmic dynein	ncd	GST/MC1	kinesin
ATP	1.03 ± 0.11	0.17 ± 0.04	0.15 ± 0.02	0.20 ± 0.03
2′-dATP	0.36 ± 0.14	0.15 ± 0.04	0.15 ± 0.04	0.066 ± 0.015
3′-dATP	0.56 ± 0.16	0.14 ± 0.03	0.11 ± 0.02	0.13 ± 0.02
dideoxy-ATP	0.36 ± 0.10	0.10 ± 0.05	0.10 ± 0.03	0.22 ± 0.05
monomethyl-ATP	0.19 ± 0.06	0.10 ± 0.03	0.14 ± 0.03	0.10 ± 0.02
dimethyl-ATP	–	–	0.11 ± 0.03	0.022 ± 0.008
PRTP	–	–	–	0.010 ± 0.004
8-bromo-ATP	–	–	–	0.005 ± 0.002
8-azido-ATP	–	–	–	0.003 ± 0.001
FTP	–	–	–	0.024 ± 0.006
etheno-ATP	–	–	0.080 ± 0.021	0.024 ± 0.011
(S_P)-ATPαS	0.18 ± 0.05	–	0.14 ± 0.04	0.034 ± 0.006
(R_P)-ATPαS	–	–	–	–
(S_P)-ATPβS	–	–	–	–
(R_P)-ATPβS	–	–	–	–
ATPγS	–	–	–	0.010 ± 0.0^3
GTP	–	–	0.048 ± 0.025	0.028 ± 0.018
ITP	–	–	0.033 ± 0.027	0.016 ± 0.006
CTP	–	–	–	0.016 ± 0.005
UTP	–	–	–	0.009 ± 0.003

[a] The in vitro motility assay was performed at 22−24 °C for ncd and at 25 °C for GST/MC1, cytoplasmic dynein, and squid optic lobe kinesin. The assay mixture consisted of 80 mM Pipes−NaOH (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, and 0.1 M NaCl for ncd and squid optic lobe kinesin, 80 mM Pipes−NaOH (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, and 0.05 M NaCl for GST/MC1, and 10 mM Tris−acetate (pH 7.5), 50 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, and 1 mM DTT for cytoplasmic dynein. The concentration of substrate was 1 mM. A dash indicates that motility was not observed over a 5 min period; at least movements faster than 0.002 μm/s were not observed with those analogues. It should be noted that with those analogues, microtubules were retained on the glass surface for any of the molecular motors used. Kinesin motility in the presence of 0.1 M NaCl was slower. In its absence, the speed with 1 mM ATP was 0.76 ± 0.06 μm/s.

be studied in the future to determine whether nucleotide specificity correlates with the direction of kinesin movement or specific subclasses of kinesin motors.

Our finding that intact ncd and GST/MC1 have different substrate specificities suggests that the load imposed on the motor may influence the nucleotide specificity profile. In this regard, the nucleotide profile of a motor in a cell-free extract assay may not be identical to that in an in vitro translocation assay. Nevertheless, a combination of nucleotide specificity and vanadate sensitivity appears to distinguish between the dynein and kinesin classes of motors. For example, dimethyl-ATP, etheno-ATP, and GTP are very poor substrates for dynein, so that any indication of motility with these analogues is likely to be indicative of a kinesin motor. Furthermore, low sensitivity to vanadate and aluminum fluoride is also a feature that so far is indicative of kinesin family members. Thus, nucleotide fingerprinting and vanadate or aluminum fluoride sensitivity remain as useful techniques for distinguishing whether kinesin or dynein-like motors are involved in reconstituted assays.

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