Reconstitution of the human cytoplasmic dynein complex

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Cytoplasmic dynein is the major motor protein responsible for microtubule minus-end-directed movements in most eukaryotic cells. It transports a variety of cargoes and has numerous functions during spindle assembly and chromosome segregation. It is a large complex of about 1.4 MDA composed of six different subunits, interacting with a multitude of different partners. Most biochemical studies have been performed either with the native mammalian cytoplasmic dynein complex purified from tissue or, more recently, with recombinant dynein fragments from budding yeast and Dictyostelium. Hardly any information exists about the properties of human dynein. Moreover, experiments with an entire human dynein complex prepared from recombinant subunits with a well-defined composition are lacking. Here, we reconstitute a complete cytoplasmic dynein complex using recombinant human subunits and characterize its biochemical and motile properties. Using analytical gel filtration, sedimentation-velocity ultracentrifugation, and negative-stain electron microscopy, we demonstrate that the smaller subunits of the complex have a significant functional requirement for complex integrity. Fluorescence microscopy experiments reveal that while engaged in collective microtubule transport, the recombinant human cytoplasmic dynein complex is an active, microtubule minus-end-directed motor, as expected. However, in contrast to recombinant dynein of nonmetazoans, individual reconstituted human dynein complexes did not show robust processive motility, suggesting a more intricate mechanism of processivity regulation for the human dynein complex. In the future, the comparison of reconstituted dynein complexes from different species promises to provide molecular insight into the mechanisms regulating the various functions of these large molecular machines.
are difficult to resolve, at least in part, because of the lack of a recombinant dynein complex with well-defined composition and the possibility of genetic manipulation.

Here, we have reconstituted a human cytoplasmic dynein complex from recombinant subunits and have characterized it biochemically. We show that the noncatalytic subunits are crucial for stable dynein heavy-chain dimerization. The reconstituted complex was an active minus-end-directed motor that did not, however, show any robust processivity.

Results
Reconstitution of the Human Cytoplasmic Dynein Complex. For complex assembly, we cloned an isoform of each of the six cytoplasmic dynein subunits, choosing a set of noncatalytic subunit isoforms that could easily be amplified from human brain cDNA (Fig. 1A). The subunits were expressed individually and then combined (Fig. 1B). The human cytoplasmic dynein heavy chain (CDHC) fused to an N-terminal oligo-histidine tag and a monomeric (m)GFP (His6-mGFP-CDHC; Fig. S1A, Top), and the IC1 were expressed in insect cells (Fig. 1C). The LIC2, LC8 light chain 1 (LC8), RB light chain 1 (RB1), and Tctex1 light chain 1 (Tctex1) were expressed as pairs of motor domains (Fig. 1A). A complex of these three proteins was purified using the His6 tag present only on the heavy chain. Three purified light chains were then added, and the mixture was subjected to gel filtration where a soluble dynein complex eluted as a broad peak (region shaded in red that followed the void-volume peak of the column in Fig. 1E). Stained SDS gels revealed that the complex contained all six dynein subunits (Fig. 1F).

To determine the oligomerization state of the purified dynein complex, we analyzed its hydrodynamic properties. Analysis by sedimentation-velocity ultracentrifugation showed that the major population of the reconstituted complex had a sedimentation coefficient of 22S (Fig. 1A, Upper), similar to the reported values for native vertebrate dynein (12S–20S; based on sucrose density gradient centrifugation analysis) (8, 15, 35). For comparison, we also purified two truncated human dynein heavy-chain constructs: the motor domain (Dyn380kD) and artificially dimerized motor domains (cc-Dyn380kD) (Fig. S1). Their sedimentation coefficients were 12S (Fig. 2A, Lower and Fig. S2A, Upper) and 19S (Fig. 2A, Lower and Fig. S2A, Lower), respectively. These results gave a first indication that a dynein complex of the correct size had formed.

To confirm this, we sought to determine the molecular mass of the reconstituted complex and the control constructs, and for this purpose, we measured their Stokes radius. Using analytical gel filtration we found that the mean Stokes radius of the reconstituted complex was 16 nm (Fig. 2B Upper and C and Fig. S2B, Inset), which was characteristically different from the Stokes radii of the controls (9 nm for the motor domain and 11 nm for the artificially dimerized motor domains) (Fig. 2B Lower and C and Fig. S2B). The molecular mass, as calculated from the sedimentation coefficient and the Stokes radius (SI Materials and Methods), was 1.6 MDa for the reconstituted complex (Fig. 2C). This confirms that it consists of a dimer of heavy chains and other subunits, like the native dynein complex (7, 15). In contrast, the human dynein motor domain alone was found to be monomeric, and the artificially dimerized motor domain was indeed a dimer, as expected (Fig. 2C).

Next, we determined the stoichiometry of subunits in the reconstituted human dynein complex. Based on quantitative analysis of SYPRO Ruby–stained SDS gels and Western blots, CDHC, LIC1, LIC2, and Tctex1 were found to be in equimolar amounts in the purified dynein complex, whereas LC8 was present in slightly higher (1.3 molecule per CDHC) and RB1 in slightly lower amount (0.6 molecule per CDHC) (Fig. 2D and Fig. S3). In conclusion, all subunits were present in the recombinant dynein complex at roughly equimolar amounts.

Low-Resolution Structure of the Reconstituted Dynein Complex. Individual dynein molecules are big enough to be observed by negative-stain EM (16). As expected, individual monomeric motor domains appeared ring-shaped (Fig. 3A) with a mean diameter of 12.9 nm (Fig. 3E), in agreement with a reported value for a recombinant dynein motor domain from Dictyostelium (18, 36). The artificially dimerized motor domains appeared, indeed, as pairs of motor domains (Fig. 3B). The reconstituted dynein complex clearly showed two identical globular motor domains connected to a tail domain (Fig. 3C). The average diameter of the motor domain was 12.2 nm, and the average length of the tail domain was 39 nm (Fig. 3F). This agreement with the dimensions of native dynein purified from tissue (7, 16). These results provide further evidence that the reconstituted human dynein complex has native properties.

Noncatalytic Subunits of the Dynein Complex Are Crucial for Heavy-Chain Dimerization and Stability. The heavy chain alone (without any accessory subunits) was mostly insoluble under our standard conditions. It eluted in the void-volume peak after gel filtration (Fig. S1B, Top, and S1C), was poorly soluble (Table 1), and appeared as large aggregates in EM (Fig. 2D). Because the dynein motor domain was highly soluble and did not aggregate (Figs. S1 and S2 and Fig. 3A), this result suggests that the tail domain of the human heavy chain aggregates in the absence of the accessory subunits and that the tail domains alone are not sufficient for correct dimerization. Increasing the ionic strength of the buffer allowed the purification of a soluble heavy chain (Fig. S4A, Lower). However, sedimentation-velocity analysis showed that under these conditions, the heavy chain also was cleared more heterogeneously than the entire dynein complex purified under the same conditions (Fig. S4A and B). Furthermore, the most prominent soluble species at high-ionic strength had a molecular mass that is rather consistent with a monomer (Fig. S4D), in clear contrast to the entire complex that also formed a dimer of heavy chains at

![Fig. 1. Reconstitution of the human cytoplasmic dynein complex. (A) Schematic of the subunit composition of the dynein complex studied here. A His6 tag, followed by mGFP (green), is fused to the N terminus of the CDHC (Fig. S1A, Top). (B) Purification scheme: lysates of cells expressing His6-mGFP-CDHC (C, lane 1; 560 kDa; referred to here as CDHC) and cells expressing IC1 (C, lane 2; 71 kDa) were mixed, supplemented with purified LIC2 (D, lane 1; 54 kDa), and subjected to immobilized metal-ion–affinity chromatography (IMAC). The eluate was supplemented with purified light chains (D, lanes 2–4; 10–13 kDa) and gel-filtered (GF). (C and D) Coomassie-stained SDS gels showing lysates of cells expressing CDHC and IC1 (C) and purified LIC2 and light chains (D), as indicated. (E) Gel-filtration profile around the position where the dynein complex elutes showing normalized absorbance values at 280 nm (black line) and 488 nm (green line). The fraction between the red lines was collected and analyzed. (F) Coomassie-stained (Left) and SYPRO Ruby–stained (Right) SDS gels showing the dynein complex after gel filtration.](https://www.pnas.org/doi/10.1073/pnas.1210573110)
increased ionic strength (Fig. S4D). No conditions were found under which the heavy chain alone formed stable, soluble dimers.

Successful dynein complex reconstitution allowed us to investigate the specific roles of the individual subunits for complex formation. Adding IC did not make the heavy chain more soluble under our standard conditions (Table 1), as demonstrated by elution of the protein in the void-volume peak after gel filtration and by the detection of mostly aggregates in EM (Fig. S5). The heavy chain in combination with LIC was partially soluble, and together with both LIC and IC, it showed similar solubility as the entire complex (Table 1). Both combinations CDHC-LIC and CDHC-IC-LIC formed mostly dimers, as demonstrated by EM (Fig. S5). However, both of these complexes did not have the entirely correct shape, as demonstrated by their reduced s values (19.5S and 19.1S, respectively) (Fig. S6B and Table 1). This was also reflected by the appearance of often less compact tails in EM images (Fig. S5). Therefore, correct complex formation requires the LIC, the IC, and the light chains, in this order of importance. These experiments identify an important structural role of the subunits for the assembly of the dynein complex.

**Motile Properties of Recombinant Dynein Constructs.** Because our dynein constructs were mGFP-tagged, we could directly observe how individual motors interact with immobilized MTs, using time-lapse total internal reflection fluorescence (TIRF) microscopy. Individual monomeric dynein motor domains showed hardly any detectable binding events in the presence of ATP and bound frequently and statically to MTs in the presence of the non-hydrolyzable ATP analog adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) (Fig. 4A and D). This is typical for a nonprocessive or weakly processive motor and is expected for truncated monomeric dynein (37). Unexpectedly, the artificially dimerized dynein motor domains exhibited similar behavior (Fig. 4B and D). In agreement with the difference in their oligomeric state (Fig. 2C and Fig. S7A), the dimers remained bound to the MT at least three times longer than the monomeric motor domain in the presence of AMP-PNP (Fig. S7B). Their mean dwell time of 3 s is in a strongly MT-bound state is, however, much shorter in comparison with that of a similar construct of processive dynein from budding yeast (38) or of processive kinesin-1 (Fig. S7C). In combination with a measured binding rate of 45 min⁻¹·NM⁻¹·µM⁻¹·MT in this experiment (Fig. 4D), which is very similar to a recently measured binding rate for

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**Fig. 2.** Oligomerization state and subunit stoichiometry of the reconstituted human dynein complex. (A, Upper) Sedimentation coefficient distribution [g (s*) plot] of the dynein complex. Gaussian functions (lines) were fitted to the data (blue circles), with the peak center at 22S for the major species (82%) of the dynein complex (red line). (A, Lower) Comparison of normalized fits to the g(s*) plots of the dynein complex (red, as above), the dynein motor domain (Dyn380kD, green; Fig. S2A, Upper), and the dynein motor domain fused to a dimerizing coiled coil (cc) sequence (cc-Dyn380kD, blue; Fig. S2A, Lower). (B, Upper) Analytical gel-filtration profile of the dynein complex. Gaussian fits (colored lines) to the data (black line) reveal a peak center at the Kav value of 0.04 (corresponding to Stokes radius, Rs, of 17 nm) for the main eluate (red line). The blue line shows the sum of the two Gaussian curves. (B, Lower) Comparison of normalized fits to the gel-filtration profiles of the dynein complex and truncated dynein constructs (color code as in A, Lower; see also Fig. S2B). (C) Summary of results from the sedimentation-velocity ultracentrifugation [s(20,w)] and analytical gel filtration (Stokes radius) and calculated partial specific volumes for the dynein complex, CDHC alone, and truncated control constructs. Mw, molecular mass. Errors are SEM. (D) Summary of the mean molar ratios of subunits in the reconstituted dynein complex. Errors are SEM. For experimental data, see Fig. S3.

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**Fig. 3.** EM images of negatively stained dynein constructs. Overview of samples (large images) and galleries of single dynein particles (small images): motor domain Dyn380kD (A); artificially dimerized motor domains cc-Dyn380kD (B); dynein complex (C); and CDHC (D). Scale is the same for all single particles images in A–D. (E) Histogram of the distribution of the Dyn380kD motor domain (MD) diameter [measured as indicated by red arrowheads in the EM image (30 × 30 nm)] (F) Histograms of the distribution of the dynein complex MD diameter (Left) and the tail domain length (Right) [measured as indicated on the EM image (60 × 80 nm) by red and yellow arrowheads, respectively]. Indicated are mean values ± SD, d, diameter; l, length.

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**Table 1.** Av values of 0.04 (corresponding to Stokes radius, Rs, of 17 nm) for the main eluate (red line). The blue line shows the sum of the two Gaussian curves. (B, Lower) Comparison of normalized fits to the gel-filtration profiles of the dynein complex and truncated dynein constructs (color code as in A, Lower; see also Fig. S2B). (C) Summary of results from the sedimentation-velocity ultracentrifugation [s(20,w)] and analytical gel filtration (Stokes radius) and calculated partial specific volumes for the dynein complex, CDHC alone, and truncated control constructs. Mw, molecular mass. Errors are SEM. (D) Summary of the mean molar ratios of subunits in the reconstituted dynein complex. Errors are SEM. For experimental data, see Fig. S3.
Table 1. Properties of dynein subcomplexes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Preparative gel filtration</th>
<th>Solubility (mg/mL)</th>
<th>s(20,W) (S)</th>
<th>CDHC oligomerization state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td>Elutes after void volume</td>
<td>~0.2</td>
<td>21.4</td>
<td>Dimer</td>
</tr>
<tr>
<td>CDHC + IC + LIC</td>
<td>Similar to the complex</td>
<td>~0.2</td>
<td>19.1</td>
<td>Dimer</td>
</tr>
<tr>
<td>CDHC + LIC</td>
<td>Similar to the complex</td>
<td>~0.1</td>
<td>19.5</td>
<td>Dimer</td>
</tr>
<tr>
<td>CDHC + IC</td>
<td>Elutes in void volume</td>
<td>~0.02</td>
<td>ND</td>
<td>Mostly aggregates</td>
</tr>
<tr>
<td>CDHC</td>
<td>Elutes in void volume</td>
<td>~0.03</td>
<td>ND</td>
<td>Aggregates</td>
</tr>
</tbody>
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ND, not determined

a dimeric dynein construct from *Dictyostelium* (23), this suggests that the affinity for MT binding of the human dynein motor domains in a strongly MT-binding state is rather low. These experiments show that, unlike its yeast and *Dictyostelium* counterparts (23, 24), the artificially dimerized recombinant dynein is unexpectedly nonprocessive, which suggests that the intrinsic motile properties of these motors are different.

Surprisingly, individual mGFP-dynein complex molecules also did not show robust processive motility (Fig. 4C), although their dimeric state in this experiment was also confirmed by single-molecule fluorescence intensity analysis (Fig. S7A). The overall behavior of the dynein complex was rather heterogeneous in the presence of ATP (Fig. 4C, Left), showing both short- and long-binding events, with many molecules staying bound for many seconds. A fraction of motors showed diffusive behavior, rarely with a directional bias. In the presence of AMP-PNP, the dynein complex bound statically, as expected, with only a small fraction of diffusively moving molecules (Fig. 4C, Right). The numerous long-binding events present in both nucleotide conditions indicate that the tail domain of the dynein complex likely contributes to binding to the MT (Fig. 4C). These data suggest that recombinant human dynein is either only weakly processive, at most, or in an inactive state.

To distinguish between these possibilities, we performed MT-gliding assays. Because our constructs had an N-terminal His tag in addition to the mGFP tag, we could immobilize the same constructs that were used for single-molecule imaging on Tris-NiNTA-PEG-functionalized glass surfaces in an oriented manner (Fig. 5A). As an ensemble, the immobilized dynein complex robustly moved polarity-marked MTs with their brightly labeled plus-ends leading (Fig. 5B and Movie S1), indicative of minus-end-directed motility. MT transport-velocity distributions were Gaussian, as expected, and the mean velocity of MT transport driven by the immobilized complex was 0.5 μm/s (Fig. 5C). Similar mean velocities were observed for the truncated monomeric and artificially dimerized dynein constructs (Fig. 5D and E and Movies S2 and S3, respectively). When the dynein complex was immobilized non-specifically to nonfunctionalized glass, the gliding speed increased by about 35% to 0.63 μm/s (Fig. 5F and Movie S4), suggesting that the orientation of the immobilized complex might have a certain influence on dynein’s motile behavior. Taken together, the gliding velocities observed here for the various recombinant human dynein constructs are close to the reported velocities of vertebrate brain-purified dynein (0.6–1.2 μm/s) (8, 35).

To estimate the degree of nonprocessivity of the human dynein complex, we measured the MT-landing rate as a function of the relative motor density (measured directly as mGFP fluorescence that increased linearly with dynein concentration). The measured landing rates predicted (39) a minimal number of three dynein complexes or artificial dimers being required to transport a MT continuously (Fig. 5G). The number for the monomer was four (Fig. 5G), very similar to the monomeric *Dictyostelium* dynein motor domain (40). These results agree with our conclusions drawn from single-dynein-molecule imaging on immobilized MTs.

In summary, recombinant human dynein complexes are active motors showing robust minus-end-directed motility when acting as an ensemble, but individual complexes are not processive.

Discussion

Here, we have demonstrated that the entire human cytoplasmic dynein complex can be assembled from six recombinant proteins (Fig. 1). The reconstituted complex has a molecular mass consistent with a heavy-chain dimer (Fig. 2A–C) and the expected shape of the native motor (Fig. 3C and F) (7, 16). All subunits are present at roughly equimolar amounts (Fig. 2D and Figs. S2 and S3), as reported previously for most subunits in the native mammalian dynein complex (9, 11, 13). The slightly lower amount of RB1 (0.6 molecule per CDHC) might be attributable to either weaker association with the IC or to an intrinsic tendency of RB1 to form isoform heterodimers, although homodimers have also been observed previously (10).

We show that the heavy-chain–associated subunits are necessary and sufficient for correct complex formation (Fig. 3C and F, Figs. S4–S6, and Table 1). Their structural importance in descending order is LIC, IC, and light chains. Full solubility requires both the presence of LIC and IC and correlates with stable heavy-chain dimerization, which is largely independent of the light chains. Nevertheless, the light chains are needed for complete folding of the complex. These results emphasize the essential structural role of the smaller dynein subunits in addition to mediating interactions with adaptor proteins and cargoes (1).

This conclusion agrees with...
previous reports about the instability of a heavy chain–LIC subcomplex obtained after separation of bovine brain–purified dynein into subcomplexes using a chaotropic agent (15) and of purified recombinant full-length rat CDHC (41). The possibility to generate a recombinant complex opens the door for a systematic dissection of the functional consequences of different subunit isoforms in the complex and for the assembly of the entire dynein/dynactin complex in recombinant form in the future.

The recombinant dynein complex is an active motor showing the expected minus-end–directed motility in MT-gliding assays (Fig. 5 B, C, and F and Movies SI and S4) (42). The measured speed of ~0.6 μm/s was well within the range of published values for native vertebrate dynein purified from tissue (8, 35) or of human recombinant dynein heavy chain overexpressed in HEK cells and purified via an affinity tag (43). This is lower than for recombinant Dictyostelium dynein (40) and higher than for yeast dynein (24), as expected. The gliding speed was not affected by the absence or presence of the tail but, to a certain extent, by the orientation of the immobilized complex can affect aspects of its motile properties.

Although the gliding assays demonstrated that the recombinant human dynein complex has expected motile properties in this assay, single-molecule fluorescence imaging showed that it is not measurably processive (Fig. 4C), and landing assays demonstrated that surface attachment also did not activate the motor in the sense of making it processive (Fig. 5G). This lack of processivity was not a consequence of a possibly not correctly assembled complex but a characteristic property of the recombinant human motor domain. This was revealed by the behavior of the truncated and artificially dimerized heavy chain (Fig. 4B) that contained the entire motor domain, including the part of the linker that was also present in the corresponding yeast and Dictyostelium constructs that were shown to be processive (23, 24). Despite its largely uncoordinated stepping mechanism, recombinant yeast dynein has been demonstrated to be a processive motor even when individual motor domains were artificially dimerized (24), probably because the individual motor domains have a sufficiently high duty ratio (fractio of time the motor is MT-bound during its biochemical cycle) in combination with a high affinity for MT binding (26, 27, 38). Similarly, artificially dimerized Dictyostelium dynein motor domains were also observed to be processive (23). However, for this motor, direct coordination between motor domains was suggested as a reason for processivity that depended on a sequence C terminus of the motor domain that is largely absent in the yeast dynein (23). This opens the possibility that different molecular mechanisms lead to processive stepping in yeast and Dictyostelium dynein. In contrast to dynein from these two species, the artificially dimerized human dynein did not show robust processive motility (Fig. 4B), as neither did the entire recombinant human dynein complex (Fig. 4C). This could be a consequence of weak MT binding of the recombinant human dynein motor domain in combination with uncoordinated stepping.

The nonprocessivity of mGFP-labeled human dynein was surprising, because most experiments with the native vertebrate dynein complex nonspecifically adsorbed to microspheres or conjugated to quantum dots by antibodies reported processive motility of individual complexes (28, 31, 32) [although nonprocessive movement has been observed as well (34)]. At present, in contrast to the yeast or Dictyostelium dynein studies, no experiments addressing the processivity of vertebrate dynein have been reported with a dynein heavy-chain fragment or a component of the dynein complex covalently attached to a fluorescent protein or small fluorophore. A study where porcine brain dynein was observed indirectly using a fluorescently labeled interaction partner (a p150 fragment not able to bind to MTs) reported an absence of...
processive runs in the presence of ATP, similar to our observations with the reconstituted human dynein (44). Native dynein from a transgenic mouse that was copurified together with the dynactin complex, carrying the fluorescent subunit S50 dispersed processively, but distinctly bidirectional movement with a biased minus-directed bias (33). This suggests that the dynactin complex might be required for processive motility of vertebrate dynein. A processivity-enhancing factor (33). This suggests that the dynactin complex might be required for processive motility of vertebrate dynein. A processivity-enhancing factor (33). This suggests that the dynactin complex might be required for processive motility of vertebrate dynein. A processivity-enhancing factor (33). This suggests that the dynactin complex might be required for processive motility of vertebrate dynein. A processivity-enhancing factor (33). This suggests that the dynactin complex might be required for processive motility of vertebrate dynein. A processivity-enhancing factor 

Materials and Methods

Proteins were expressed in E. coli or in insect cells and purified using a combination of metal ion-affinity chromatography and gel filtration. The oligomeric state of the dynein constructs and the subunit stoichiometry of the complex were determined using analytical ultracentrifugation, analytical gel filtration, negative stain electron microscopy, gel electrophoresis and western blotting. The motile properties of dynein were measured in TIRF-microscopy-based motility assays. For details, see SI Materials and Methods.

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10. Nikulina K, Patel-King RS, Takebe S, P (2005) ATP hydrolysis cycle-de-
Supporting Information

Troker et al. 10.1073/pnas.1210573110

SI Materials and Methods

Cloning and Expression of Dynein Heavy-Chain Constructs and Associated Subunits. Full-length human cytoplasmic dynein 1 heavy chain 1 (DYNC1H1; UniProtKB accession no. Q14204) cDNA encoding 4,646 aa was generated by producing three fragments that were subsequently combined and inserted into a modified baculoviral expression vector with inserted mGFP (pFastBacHT-mGFP), generating His6-mGFP-CDHC (CDHC).

In detail, three fragments covering the entire full-length CDHC cDNA were first generated. A cDNA encoding the 3′ fragment (encoding amino acids 1947–4646 of the protein) was obtained from the cDNA clone DKFZp626G1167 (ImaGenes; GenBank accession no. AL833600.1) and amplified by PCR. Mutations present in the cDNA clone (R3339H, F3354L, K3774X, L3863P) were reverted to the National Center for Biotechnology Information (NCBI) reference sequence NM_001376 using site-directed mutagenesis (Strategene). The 5′ fragment (encoding amino acids 1–1190) and the central fragment (encoding amino acids 1187–1970) were generated by RT-PCR of mRNA isolated from HeLa cells using gene-specific primers. The amplified fragments were first cloned into PCR-XL-TOPO vector (Invitrogen). For the ligation of the 5′- and central cDNA fragment, an existing SacI restriction site was used. For the ligation of the central and the 3′ fragment, we introduced a SacII restriction site without changing the amino acid composition of the protein (at amino acid position 1969–1970). For the ligation of the 5′ end of the CDHC cDNA, a MluI site was introduced into the multiple cloning site (MCS) of pFastBacHTa, and for the ligation of the 3′ end, the existing SpeI site in MCS was used. A sequence encoding mGFP (the K221L mutation of eGFP) (1) was inserted before the MluI site in the modified pFastBacHTa, generating pFastBacHT-mGFP. Full-length CDHC was assembled and cloned into pFastBacHT-mGFP by simultaneous ligation of four fragments (three CDHC cDNA fragments and the vector), generating an expression vector encoding for His6-mGFP-CDHC, with amino acids GGGYASGA linking mGFP and CDHC.

A sequence encoding the C-terminal 380-kDa dynein motor domain (1,303–4,646 aa) was amplified from His6-mGFP-CDHC and cloned into the pFastBacHT-mGFP vector, generating His6-mGFP-Dyn380kD, with amino acids GGGYASGGTGAGAAA linking mGFP and Dyn380kD.

An artificial dimer of dynein motor domains was generated by fusing the sequence encoding for a kinesin-1 coiled coil to the 5′ end of the dynein motor domain. The coiled coil fragment (cc) (encoding kinesin-1, amino acids 337–414) was amplified from the human kinesin-1 heavy-chain full-length cDNA clone IRCP5012D0817D from ImaGenes (UniProtKB accession no. P33176) and cloned into pFastBacHT-mGFP-Dyn380kD, generating His6-mGFP-cc-Dyn380kD, with amino acids GGGYASGG linking mGFP and cc, and amino acids GSGGGGAA linking cc and Dyn380kD.

The accessory subunits of the dynein complex were amplified from a first-strand cDNA library from human brain (Origene) by PCR using primers suitable to amplify any of the respective isoforms. We obtained sequences for human cytoplasmic dynein 1 IC1 isoform 2 (DYNC111; UniProtKB accession no. O14576-2; 628 aa), human cytoplasmic dynein 1 LIC2 (DYNC1L2; UniProtKB accession no. O43237; 492 aa), human cytoplasmic dynein LC8 light chain 1 (DYNL1L1; UniProtKB accession no. P63167; 89 aa), human dynein RB light chain 1 (DYNLRB1; UniProtKB accession no. Q9NP97; 96 aa), and human dynein Tctex1 light chain 1 (DYNLTI1; UniProtKB accession no. P63172; 113 aa). All five subunits [IC1, LIC2, and the light chains (LC8, RB1, and Tctex1)] were cloned into a bacterial expression vector pETZ22, generating His6-Ztag proteins, containing a tobacco etch virus (TEV) protease cleavage site between Z tag and the protein. IC1 was additionally cloned into a baculoviral expression vector pFastBacET (version of pFastBac 1 with modified MCS), generating a construct without additional tags.

The accuracy of each cloned sequence described above was confirmed by sequencing.

pFastBac vectors were used to generate recombinant baculoviruses for insect cell expression, essentially as described (2). All of the baculoviruses were titered and used in a dilution that allows the infected SF21 insect cells to divide once before cell-proliferation arrest (low multiplicity of infection). His6-mGFP-CDHC and IC1 were expressed for 60 h after proliferation arrest, whereas His6-mGFP-Dyn380kD and His6-mGFP-cc-Dyn380kD were expressed 72 h after the arrest. After the expression, the cells were harvested and frozen in liquid nitrogen.

Protein Purification. Dynein heavy-chain constructs. Cells expressing either full-length CDHC or truncated dynein construct (His6-mGFP-CDHC, His6-mGFP-Dyn380kD, or His6-mGFP-cc-Dyn380kD) were lysed in ice-cold lysis buffer A [50 mM HEPES, 250 mM potassium acetate (KOAc), 2 mM MgSO4, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 30 mM imidazole (pH 7.4), protease inhibitors (Complete EDTA-free; Roche)] supplemented with 0.5 mM EDTA using a glass homogenizer (Fisher Scientific). The lysate was clarified by centrifugation at 250,000 × g for 30 min at 4 °C and applied to 1-mL HisTrap HP column (IMAC; GE Healthcare) equilibrated in lysis buffer A. The column was then washed extensively with lysis buffer A and the protein was eluted with elution buffer A [30 mM HEPES, 150 mM KOAc, 2 mM MgSO4, 0.25 mM EDTA, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 500 mM imidazole (pH 7.4), protease inhibitors] using a linear gradient of 10 mM. Dynein-containing fractions were pooled, and the buffer was exchanged into gel-filtration buffer A [30 mM HEPES, 150 mM KOAc, 2 mM MgSO4, 0.5 mM EGTA, 10% glycerol (vol/vol), 0.1 mM Mg-ATP, 10 mM β-mercaptoethanol (pH 7.2)] using a PD10 desalting column (GE Healthcare). The protein was gel-filtered using a Superose 6 10/300 GL column (GE Healthcare) equilibrated in gel-filtration buffer A and concentrated. [The absorbance values at 280 and 488 nm of mGFP-labeled dynein constructs indicated that there was roughly one mGFP per each dynein monomer. There was also a significant fraction of His6-mGFP in the eluate that was well separated from the dynein construct (Fig. S1B,Inset) and was probably a consequence of prematurely terminated translation.] After centrifugation at 100,000 × g for 10 min at 4 °C, the protein concentration was determined by measuring the absorbance at 280 nm and using a molar extinction coefficient calculated from its primary sequence (Expsys; http://expasy.org/tools/protparam.html). The concentration was confirmed by Bradford using BSA as a standard. Proteins were supplemented with glycerol to a final concentration of 20% (vol/vol), flash-frozen in small aliquots, and stored in liquid nitrogen. The final yields of purified Dyn380kD and cc-Dyn380kD were typically ~2 mg from 500 mL of SF21 cell culture, with a final concentration up to ~1 mg/mL. In contrast, purified CDHC could not be concentrated beyond ~0.03 mg/mL.

Mass spectroscopic analysis of the artificially dimerized dynein confirmed that the entire amino acid sequence of this construct

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could be detected (with an amino acid coverage of the sequence of 66%), demonstrating that the purified protein was intact.

For the analysis of hydrodynamic properties and oligomerization state under higher-ionic-strength conditions (in Fig. S4), dynein constructs were purified using purification buffers of higher ionic strength: lysis buffer B [50 mM KH₂PO₄, 300 mM KCl, 1.2 mM MgCl₂, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 30 mM imidazole (pH 7.4), protease inhibitors], elution buffer B [50 mM KH₂PO₄, 500 mM KCl, 2 mM MgCl₂, 0.25 mM EDTA, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 500 mM imidazole (pH 7.4), protease inhibitors], and gel-filtration buffer B [20 mM KH₂PO₄, 250 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, 10% glycerol (vol/vol), 0.1 mM Mg-ATP, 10 mM β-mercaptoethanol (pH 7.2)].

Heavy-chain-associated subunits. pETZT2 constructs (IC1, LIC2, LC8, RB1, and Tctex1) were used to transform E. coli BL21 RIL cells, and the culture was grown from a single clone to an OD₆₀₀ of 0.6. Protein expression was induced by adding 0.1 mM isopropylthio-β-galactoside (IPTG), and the culture was incubated overnight at 18 °C shaking at 200 rpm. Cells were harvested and lysed in lysis buffer C [50 mM NaH₂PO₄, 300 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 5 mM imidazole (pH 7.5)] supplemented with protease inhibitors (Complete EDTA-free; Roche) using a high-pressure homogenizer (EmulsiFlex C5; Avestin). The cell lysate was clarified by centrifugation at 250,000 × g for 30 min at 4 °C and applied to a 5-mL HiTrap chelating HP column (GE Healthcare) loaded with cobalt ions and equilibrated with lysis buffer C. The column was then washed extensively with lysis buffer C, and the protein was eluted with lysis buffer C supplemented with 350 mM imidazole. The eluted protein was dialyzed into lysis buffer C and incubated overnight at 4 °C with oligo-histidine-tagged TEV protease (1 mg of protease per 30 mg of substrate). Cleaved His₆-Z tag and the protease were removed by rebinding to a cobalt-charged HiTrap chelating HP column. The flow-through (containing cleaved proteins) was concentrated and further purified by gel filtration using a HiLoad 16/60 Superdex 200-20 column equilibrated in gel-filtration buffer C [20 mM NaH₂PO₄, 250 mM KCl, 2 mM MgCl₂, 5 mM β-mercaptoethanol (pH 7.2)]. The protein concentration was determined by Bradford using BSA as a standard. Proteins were supplemented with glycerol to a final concentration of 30% (vol/vol), flash-frozen in liquid nitrogen, and stored in aliquots.

IC1 was the only accessory subunit that was insoluble after the purification process and could be detected (with an amino acid coverage of the sequence of 75%), demonstrating that the purified protein was intact.

For the analysis of hydrodynamic properties and oligomerization state under higher-ionic-strength conditions (in Fig. S4), dynein constructs were purified using purification buffers of higher ionic strength: lysis buffer B [50 mM KH₂PO₄, 300 mM KCl, 1.2 mM MgCl₂, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 30 mM imidazole (pH 7.4), protease inhibitors], elution buffer B [50 mM KH₂PO₄, 500 mM KCl, 2 mM MgCl₂, 0.25 mM EDTA, 10% glycerol (vol/vol), 0.2 mM Mg-ATP, 10 mM β-mercaptoethanol, 500 mM imidazole (pH 7.4), protease inhibitors], and gel-filtration buffer B [20 mM KH₂PO₄, 250 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, 10% glycerol (vol/vol), 0.1 mM Mg-ATP, 10 mM β-mercaptoethanol (pH 7.2)].

Reconstitution of dynein complex. To assemble and purify the cytoplasmic dynein complex, consisting of CDHC, IC1, LIC2, and three light chains, we first preassembled a ternary complex. The heavy chain and IC1 (both expressed in insect cells) were not purified before complex assembly, because in contrast to the other subunits, they were insoluble when purified individually. Four grams of cells expressing His₆-mGFP-CDHC (~1–2 mg expressed CDHC), 2.35 nmol and 1 g of cells expressing IC1 (~5–10 mg expressed IC1; 70–140 nmol) were resuspended in ice-cold lysis buffer A supplemented with 0.5 mM EDTA. The cell suspensions were mixed and lysed using a glass homogenizer. Purified LIC2 (2 mg; ~35 nmol) was added to the lysate, and this mixture was centrifuged at 250,000 × g for 30 min at 4 °C. Clarified lysate was applied to 1-mL HisTrap HP column equilibrated in lysis buffer B. The column was then washed extensively with lysis buffer B, the protein was eluted with elution buffer A using a linear gradient of 10 mM Mg-ATP and dynein-containing fractions were pooled. This first purification step removed excess IC1 and LIC2, and the obtained ternary complex is the minimal complex showing good solubility (Table 1). Then, 150 μg (~10 nmol) of each purified light chain (LC8, RB1, and Tctex1) were added to the eluate. The buffer of this protein mixture was immediately exchanged into gel-filtration buffer A using a PD10 desalting column, and the sample was gel-filtered using a Superose 6 10/300 GL column equilibrated in gel-filtration buffer A. The fractions following the void-volume peak of the column, as indicated in Fig. 1E, were collected. The solution was concentrated and centrifuged at 100,000 × g for 10 min at 4 °C. The extinction coefficient and molecular mass that were used for calculation of the concentration of the complex in the supernatant were obtained by adding the molar extinction coefficients and molecular masses, respectively, of the six subunits calculated from their primary sequences (ε = ~820,000 M⁻¹ cm⁻¹, molecular mass, 723 kDa). The purified dynein complex was supplemented with glycerol to a final concentration of 20% (vol/vol), flash-frozen in small aliquots, and stored in liquid nitrogen. The final yield of the purified dynein complex was ~0.2–0.3 mg of protein, with a final concentration of ~0.2 mg/mL.

To test the role of different accessory subunits in dynein heavy-chain assembly, we purified cytoplasmic dynein subcomplexes with different subunit combinations by leaving out one or more accessory subunits during the reconstitution and purification protocol. The protein concentration that could be reached with these purified subcomplexes was determined from the SYPRO Ruby-stained SDS gel-band intensities of their heavy chain using purified complex as a calibration standard. This allowed comparison of the solubility of these partial complexes with the complete complex.

For purification of the dynein complex under higher-ionic-strength conditions, lysis buffer B, elution buffer B, and gel-filtration buffer B were used. Tubulin purification and labeling. Pig brain tubulin was purified as described previously (3) and labeled with Alexa Fluor 568 N-hydroxysuccinimide (NHS) (Invitrogen) or biotin–NHS (Thermo Fisher Scientific) (4). Tubulin concentrations refer to dimers.

Antibody production. Polyclonal antibody against cytoplasmic dynein light chain RB1 (α-RB1 antibody) was generated by immunizing rabbits (Eurogentec) with the purified recombinant RB1. Rabbit antiserum was purified by antigen-specific affinity chromatography using purified RB1 covalently coupled to HiTrap NHS-activated HP column (GE Healthcare) following standard procedures. The antibody concentration was determined by Bradford using BSA as a standard, and the purity was analyzed by SDS/PAGE. Purified polyclonal antibody was stored in small aliquots at −20 °C in PBS containing 50% (vol/vol) glycerol.

Other proteins. Monomeric and dimeric kinesin-mGFP containing either the first 340 (Kin340-mGFP) or 401 (Kin401-mGFP) amino acids of the Drosophila melanogaster kinesin-1 heavy chain, respectively, fused to mGFP were expressed and purified as described previously (5, 6).

Sedimentation-Velocity Analytical Ultracentrifugation. Sedimentation-velocity ultracentrifugation was used to determine the sedimentation coefficients of dynein constructs. For sedimentation-velocity runs in standard buffer conditions, proteins were purified by IMAC (as described above) using lysis buffer A and elution buffer A and then gel-filuted into analytical ultracentrifugation (AUC) buffer A [30 mM Hepes, 150 mM KCl, 2 mM MgSO₄, 0.68 M glycerol, 2 μM Mg-ATP, 0.2 M TCEP (Tris(carboxymethyl)phosphine; Sigma) (pH 7.0)]. For measuring the sedimentation coefficients under higher-ionic-strength conditions (Fig. S4B), proteins were purified by IMAC using lysis buffer B and elution buffer B and then gel-filuted into AUC buffer B [20 mM KH₂PO₄, 250 mM KCl, 2 mM MgCl₂, 0.68 M glycerol, 2 μM Mg-ATP, 0.2 M TCEP (pH 7.2)]. For each sedimentation-velocity run, 400 μL of protein sample was analyzed. The dynein complex, CDHC-IC-LIC, and CDHC were prepared at ~200 nM concentrations and CDHC-LIC at ~100 nM, whereas Dyn380kD
and cc-Dyn380kD were analyzed at concentrations ranging from 300 nM to 1.2 μM.

Sedimentation-velocity ultracentrifugation was performed in a Beckman analytical ultracentrifuge (model Optima XLA) equipped with an UV-absorption optical system and an An-Ti50 rotor. Experiments were done in double-sector charcoal-Epon cells at 20 °C at 30,000 or 40,000 rpm. Scans of absorbance at 230 or 280 nm were recorded using a spacing of 0.003 cm in a continuous scan mode. Absorbance scans were taken at 230 nm when the concentration of the sample was lower than 400 nM; otherwise, the scans were collected at 280 nm. Data were analyzed with DCDT+ (Version 2.3.2), using the refined version of time-derivative method (7). The density and viscosity of each sample buffer and the partial specific volume of each protein were calculated using SEDNTERP (Version 1.09). The Gaussian function was fitted to sedimentation coefficient distributions to obtain the sedimentation coefficients of each protein species.

Analytical Gel Filtration. Stokes radii of dynein constructs were measured by gel-filtration chromatography using a Superose 6 PC 3.2/30 column (GE Healthcare) equilibrated in AUC buffer A and using protein standards of known Stokes radii for calibration [Gel Filtration Calibration Kits LMW and HMW (GE Healthcare): thyroglobulin (8.5 nm), ferritin (6.1 nm), aldolase (4.81 nm), ovalbumin (3.05 nm), and ribonuclease A (1.64 nm)]. Dynein constructs were prepared as described for analytical ultracentrifugation experiments. For each gel-filtration experiment, 50 μL of protein sample was analyzed. Elution volumes ($V_e$) were measured by fitting a Gaussian function to the elution peaks using OriginPro (Version 8.6). The elution volume parameter $K_{av}$ was calculated for each protein using

$$K_{av} = \frac{V_e - V_0}{V_C - V_0}$$

where $V_C$ is the geometric column volume (2.4 mL for a Superose 6 PC 3.2/30), and $V_0$ is the column void volume (determined from the elution volume of Blue Dextran 2000). The calibration curve

$$\sqrt{-\log(K_{av})} = aR_s + b$$

was used to calculate the Stokes radii ($R_s$) of the dynein constructs.

For measuring Stokes radii under higher-ionic-strength conditions (Fig. S4C), analytical gel filtration was carried out as described above, but both the protein standards and dynein constructs were separated on a column equilibrated with AUC buffer B.

Molecular Mass Determination. The molecular masses of dynein constructs were calculated using the experimentally determined Stokes radii and sedimentation coefficients using the Svedberg equation:

$$M = \frac{6\pi \eta N R_s s}{1 - v_p}$$

where $M$ is molecular mass, $\eta$ is viscosity of the water at 20 °C (0.01 g-cm⁻¹-s⁻¹), $N$ is Avogadro's number (6.022 × 10²³), $R_s$ is Stokes radius of the protein (nanometers), $s$ is a sedimentation coefficient of the protein corrected to standard conditions [at 20 °C in water: s(20,ω); 10⁻¹³ s (S)], $v$ is partial specific volume of the protein (cubic centimeter per gram), and $\rho$ is density of the water at 20 °C (1 g-cm⁻³).

Subunit Stoichiometry Determination in the Reconstituted Dynein Complex. Five out of six subunits in the purified dynein complex were analyzed by quantifying their band intensities of SYPRO Ruby–stained (Molecular Probes) SDS gels using protein standards of known concentration for calibration. The amount of dynein heavy chain (CDH): 560 kDa per monomer) and dynein IC (IC1: 71 kDa) in the complex was estimated using Dyn380kD (413 kDa) and BSA (Sigma; 69 kDa), respectively, of known concentration as a standard. The SYPRO Ruby–stained SDS gels were scanned [with a range of exposure times (0.25–4 s), depending on the subunit analyzed] using ImageQuant LAS 4010 (GE Healthcare), and the intensity of the bands of each protein was determined using ImageQuant TL software. Subunit stoichiometry is presented as a ratio of calculated amounts of individual subunits.

Because the LC8 light chain was not well separated by SDS/PAGE from RB1 and was not well stained with SYPRO Ruby, LC8 and RB1 were also quantified by Western blotting, using purified proteins of known concentration as a standard. We determined the ratio of LC8 and RB1 light chains per LIC2 in the dynein complex, by quantifying LIC2 from the same gel using SYPRO Ruby for staining. In detail, serial dilutions of standards (RLC2 and Dyn380kD) and purified dynein complex were run on an SDS gel. The gel was then cut horizontally at the 25-kDa marker. The upper part of the gel was stained with SYPRO Ruby gel stain, and the amount of LIC2 was quantified as described above. The lower part of the gel was wet-blotted on a nitrocellulose membrane with 0.1-μm pore size (Whatman), and the blot was blocked using Odyssey blocking buffer (Licor). LC8 was detected using polyclonal rabbit α-LC8 antibody (ab87283; Abcam), and RB1 was detected using α-RB1 antibody. The primary antibodies were detected using IR secondary antibody (IRDye 680LT goat anti-rabbit IgG; Licor), and the blots were scanned and analyzed using Licor Odyssey Infra-red Imagier.

Negative-Staining EM. Purified dynein constructs were diluted in 30 mM Hapes, 150 mM KOAc, 2 mM MgSO₄, 0.1 mM ATP, and 10 β-mercaptoethanol (pH 7.2) at ~3 nM final concentration. A total of 4 μL of the sample solution was applied to a carbon-coated grid (Plano), previously activated by glow discharge. After 2 min, the grid was washed quickly three times with water and four times with 2% uranyl acetate (wt/vol), blotting the liquid away after each step and leaving the grid to air-dry after the last step. Samples were imaged with a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company) at a voltage of 120 kV and an Ultrascans CCD (Gatan). The diameter of dynein motor domains and the length of the dynein complex tail were measured using ImageJ.

MT-Gliding Assays. MT-gliding assay on Tris-Ni-NTA surface. Tris-NTA-PEG–functionalized glass coverslips were prepared and loaded with nickel ions as described previously (8). Flow chambers were built from a Tris-Ni-NTA-PEG coverslip and poly(t-lysine) (PLL)-PEG–passivated counter glass, separated by two strips of double-sided sticky tape (Tesa). Taxol-stabilized and polarity-marked MTs were prepared as described previously (8, 9). The flow chamber was additionally passivated by incubation with 5% Pluronic F-127 in water (wt/vol) for 5 min and then placed on an ice-cold metal block and washed with immobilization buffer (gel-filtration buffer A). His₆-motor diluted in immobilization buffer at concentrations between 1 and 500 nM was incubated for 30 min. Unbound proteins were washed off with immobilization buffer, followed by washing with assay buffer (AB) [10 mM Pipes, 35 mM KOAc, 2 mM MgSO₄, 1 mM EGTA, 2 mM β-mercaptoethanol, 10 μM Taxol (pH 7.0)]. The flow chamber
was then allowed to warm up to room temperature, and AB supplemented with 2 mM Mg-ATP, oxygen scavenger system [20 mM glucose, 20 μg/mL catalase (Sigma), and 160 μg/mL glucose oxidase (Serva)], and 50–300 nM Alexa568-labeled MTs was flowed into the chamber. MTs were immediately visualized at 25 ± 1 °C by shuttered time-lapse TIRF microscopy [IX71 (Olympus); using a setup as described (6)], with a 60x objective. Samples were excited at 532 nm, and images were acquired at 1-s time intervals with 100-ms exposure times. For landing-rate experiments, five consecutive images of the sample (and of a control sample lacking motor) were also taken with 488-nm excitation and with 30- to 400-ms exposure time (depending on the concentration used for immobilization). Movies S2, S3, and S4 have been compressed to reduce file size.

**MT-gliding assay on nonfunctionalized glass.** Flow chambers were built from untreated glass coverslips and PLL-PEG–passivated counter glass. The flow chamber was passivated by incubation with 5% Pluronic F-127 in water (wt/vol) for 5 min and was washed with immobilization buffer. Dynemin complex diluted in immobilization buffer at concentrations between 5 and 150 nM was flowed into a chamber and incubated for 3 min, followed by 1 min of incubation with 5 mg/mL β-casein in immobilization buffer. Unbound proteins were washed off with immobilization buffer, followed by washing with AB and AB supplemented with 2 mM Mg-ATP, oxygen scavenger system (same as described above), and 50–300 nM Alexa568-labeled MTs were flown in the chamber, and MTs were immediately visualized in the same way as described above.

**Kymograph analysis of gliding assays.** To determine the velocities of MT gliding from a time-lapse movie, the tracks of gliding MTs were analyzed with the “Multiple Kymograph” plug-in for ImageJ (J. Rietdorf and A. Seitz, EMBL, Heidelberg, Germany). Velocities were determined from kymographs (time-space plots) for intervals of 1–2 min. Approximately 100 MTs were analyzed per condition. A Gaussian function was fitted to gliding velocity histograms using OriginPro. The reported mean velocity is the center of the peak of the fitted Gaussian function and the reported error is SD.

**Landing-rate assay analysis.** To determine the motor density on the surface, we measured the average background-corrected mGFP intensity from the images taken with 488-nm excitation. The exposure time was chosen to avoid signal saturation; the measured intensities were then normalized with exposure time after ensuring a linear dependence. Variations in excitation intensities on different days were corrected for by using fluorescent beads as calibration standards.

The landing rate for each motor density was calculated by measuring the number of MTs within a field of view (18,700 μm²) that landed within 1–5 min (depending on motor density and MT concentration) and moved at least for three consecutive frames (corresponding to 0.8 μm). The landing rates were corrected for different MT concentrations after ensuring the rates depended linearly on the MT concentration. In the absence of immobilized motors, no MTs landed on the surface.

To quantify the minimal number n of motors required for MT transport, the dependence of the MT landing rate, Ln, was plotted as a function of the motor density, ρ, and the following function was fitted to the data, as previously described (10):

\[ Ln(\rho) = A \cdot e^{-n/\rho} \]

The fit was performed using logarithmic values of landing rates. We confirmed that the determination of the minimal motor numbers required for MT transport was not affected by potential changes in the length distribution of the landing MTs with changing motor density.

**Single-Molecule Imaging Assay.** Individual mGFP-labeled motors were imaged at low concentrations on immobilized fluorescently labeled MTs using TIRF microscopy (8). Flow chambers were built from biotin–PEG–functionalized coverslips and PLL-PEG–passivated counter glass. The flow chamber was additionally passivated by incubation with 5% Pluronic F-127 in water (wt/vol) for 5 min on an ice-cold metal block, followed by washing with 50 μg/mL χ-casein (Sigma) in AB. To enable MT immobilization, 50 μg/mL χ-casein and 100 μg/mL NeutrAvidin (Invitrogen) in AB were incubated for 5 min, and the flow chamber was washed with 50 μg/mL χ-casein in AB. The flow chamber was then allowed to warm up to room temperature, and ~40 nM biotinylated and Alexa568-labeled MTs in AB were flown into the chamber and incubated for 5 min. Nonbound MTs were washed out with AB, and, finally, AB containing a nucleotide [2 mM Mg-ATP, unless otherwise stated; or 1 mM Mg-AMP-PNP (Sigma)], oxygen scavenger system (20 mM glucose, 20 μg/mL catalase, and 160 μg/mL glucose oxidase), and mGFP-motor (at concentrations as indicated in the figure; typically 50–100 pM mGFP–dynemin complex, 30–500 pM mGFP-Dyn380kD, or mGFP-cc-Dyn380kD, and 10 pM of Kin401-mGFP) was flowed into the chamber. Motors were immediately visualized by TIRF microscopy (100x objective; 1.6x optovar) and recorded by continuous streaming in the mGFP channel (488-nm excitation; 100-ms exposure time) for 70 s, followed by generation of a static image of MTs averaging a continuous stream of 10 images (532-nm excitation; 100-ms exposure time). The temperature was maintained at 25 ± 1 °C.

To determine the behavior of single fluorescent motors on the MTs, 90–95% of all events (for 15%, either landing or dissociation was not detected, because it happened before the start or after the end of the movie, respectively), and the percentage of the mGFP-cc-Dyn380kD complete events was ~80% of all events (for 15%, either landing or dissociation was not detected, and 5% were continuously bound from the beginning to the end of the movie). The cumulative distribution function (cdf) of data were calculated for the complete events using the “ecdf” function in Matlab (R2011b), and a monoequivalent function was fitted to (1 – cdf) data using OriginPro:

\[ 1 – cdf = A \cdot e^{t} \]

with amplitude (A), dwell time (t), and mean dwell time (τ). Reported errors are SEM.

**GFP fluorescence intensity analysis of single molecules.** To measure fluorescence intensities of single molecules, mGFP-fused motors at 40–80 pM concentration, diluted in AB supplemented with 2 mM Mg-ATP and oxygen scavenger system (20 mM glucose, 375 μg/mL glucose oxidase, 90 μg/mL catalase) were nonspecifically.
adsorbed onto a glass surface and imaged by continuous streaming with either 400-ms exposure time for 20 s (Fig. S7A, Upper) or with 100-ms exposure time for 5 s (Fig. S7A, Lower) using TIRF microscopy. For the intensity distribution analysis, the raw image stack was imported into ImageJ, and the central two-thirds of each image was selected for analysis. The “GaussianFit” plug-in ([http://valelab.ucsf.edu/~nico/IJplugins](http://valelab.ucsf.edu/~nico/IJplugins)) was used to measure the integrated intensity of individual fluorescence signals, by fitting 2D Gaussian profiles to every molecule in each frame of the image stack. The fitting parameters and background were automatically calculated using 15 × 15 pixel regions around each molecule. The fitting results were imported into Origin 8.5 and were filtered to remove obviously spurious data, based on an estimated width of 220 nm for the optical point-spread function of the microscope. Relative frequency histograms of the integrated intensity were calculated; this was repeated for four to five different image stacks for each construct assayed, and the histograms were averaged.

Fig. S1. Human cytoplasmic dynein heavy chain (CDHC) constructs used in this study. (A) Schematic of the domain organization of the dynein heavy-chain sequences used here. All constructs carry a N-terminal His$_6$ tag and a mGFP (green): full-length CDHC, monomeric cytoplasmic dynein motor domain (Dyn380kD), and artificially dimerized cytoplasmic dynein motor domains (cc-Dyn380kD), generated by fusion of kinesin-1 coiled coil (cc) to the N terminus of the dynein motor domain. (B) Preparative gel-filtration profiles of the three constructs with normalized absorbance values at 280 nm (black line) and 488 nm (green line) in the region around the dynein elution peaks. CDHC purified in the absence of its accessory subunits showed poor solubility throughout the purification and eluted in the void-volume peak, suggestive of protein aggregation. The collected CDHC fraction (red shaded area) was rather insoluble (Table 1). In contrast, truncated dynein constructs Dyn380kD and cc-Dyn380kD were highly soluble and eluted as a single peak after the void-volume peak during gel filtration, showing no signs of aggregation. This result strongly suggests that the tail domain of human CDHC is not sufficient for heavy-chain dimerization, but rather causes aggregation in the absence of the other subunits of the dynein complex. (Inset) Elution profile over an extended range. All preparations contained truncated mGFP that eluted after dynein and could be well separated. (C) Coomassie-stained SDS gel of purified Dyn380kD, cc-Dyn380kD, and CDHC.
Fig. S2. Hydrodynamic properties of dynein motor domain Dyn380kD and artificially dimerized motor domains cc-Dyn380kD. This figure supplements Fig. 2 A–C. (A) Sedimentation coefficient distributions \([g(s^*)]\) plots of Dyn380kD (Upper) and cc-Dyn380kD (Lower) as obtained from analytical sedimentation-velocity ultracentrifugation. Gaussian fits (lines) to the data (blue circles), with the center being at 12S for the major species (85%) of Dyn380kD (green line) and 19S for the single species of cc-Dyn380kD (blue line). The gray and blue lines in the upper plot represent a minor population and the sum of two species of Dyn380kD, respectively. (B) Analytical gel-filtration profiles of Dyn380kD (Upper) and cc-Dyn380kD (Lower) with absorbance values at 280 nm. Gaussian fits (colored lines) to the data (black lines), with the center of the peak corresponding to \(K_{av}\) values of 0.26 for Dyn380kD (green line) and 0.18 for cc-Dyn380kD (blue line). Corresponding Stokes radii, \(R_s\), are as indicated. (Inset) Gel-filtration calibration curve for the determination of Stokes radii. The plot shows \(\sqrt{-\log(K_{av})}\) values of protein standards as a function of the known Stokes radii (blue diamonds) and a linear fit (red line). Shown are SEM. 1, RNase A; 2, ovalbumin; 3, aldolase; 4, ferritin; 5, thyroglobulin. Adjusted \(R^2 = 0.992\).
Fig. S3. Quantification of the subunit stoichiometry in the reconstituted dynein complex. This figure supplements Fig. 2D. (A) SYPRO Ruby–stained SDS gel with serial dilutions of a mixture of calibration proteins [Dyn380kD, BSA, and LIC2 (S1–S5) at known concentrations] and of the reconstituted dynein complex (C1–C5; with CDHC, IC1, and LIC2 labeled). (B) Calibration curves of gel-band intensities of Dyn380kD (red), BSA (blue), and LIC2 (green) (as measured in the calibration protein mixtures S1–S5 in A) as a function of the protein amounts loaded per lane. (C) Table showing the amounts of the subunits in the dynein complex (as detected in A and calculated using the calibration curves shown in B) and the corresponding molar ratio of subunits. Note: smaller subunits were below the detection limit in lanes S4 and S5 and C4 and C5. (D) Western blot with serial dilution of RB1 and LC8 light chains of known concentrations and of the dynein complex (probed against RB1 and LC8 light chains). (E) Calibration curves of Western blot band intensities of the purified protein standards (RB1 in red, LC8 in green) as a function of protein amounts loaded per lane. (F) Table with the determined amounts of subunits in the dynein complex and the corresponding molar ratio of subunits. RB1 and LC8 amounts were calculated using the calibration curves shown in E, and the amounts of LIC2 were calculated from the SYPRO Ruby–stained upper part of the SDS gel that was not used for blotting, using the LIC2 calibration curve (as shown in A–C).
Fig. S4. Purification and oligomerization state of dynein constructs in higher-ionic-strength conditions. Dynein constructs in this experiment were both purified and analyzed in high-ionic-strength buffer (SI Materials and Methods). (A) Preparative gel-filtration profiles of the dynein complex (Upper) and the CDHC (Lower) in high-ionic-strength buffers showing normalized absorbance values at 280 nm (black line) and 488 nm (green line). Purifying CDHC under these conditions resulted in the appearance of a second CDHC peak (in addition to the void-volume peak) compared with standard conditions (Fig. S1B, Top). Red lines indicate the fractions that were collected and further analyzed by sedimentation-velocity ultracentrifugation (B) and analytical gel filtration (C). (B) Analytical ultracentrifugation: sedimentation coefficient distributions [g(s*) plots] of the dynein complex (Upper) and the CDHC (Lower) in high-ionic-strength buffer. Gaussian fits (lines) to the data (blue circles) with the center at 20S for the dynein complex (red line), 16S for the lower s-value species of CDHC (brown line; 46%), and 38S for the higher s-value species (gray line; 54%). These results show that even under these conditions, the CDHC sample is more heterogeneous than the dynein complex. (C) Analytical gel-filtration profiles of the dynein complex (Upper) and the CDHC (Lower) (with absorbance values as indicated). Gaussian fits (colored lines) to the data (black lines) with the center of the peak corresponding to a Kav value of 0.08 for the dynein complex (red line) and 0.23 for the main eluate of the CDHC (brown line). Stokes radii, Rs, are as indicated. For comparison: the Kav values for cc-Dyn380kD and Dyn380kD under the same conditions were 0.25 and 0.34, respectively. (D) Summary of results from the analytical sedimentation-velocity ultracentrifugation [s(20,w)] and analytical gel filtration (Stokes radius). The oligomerization state was determined as the ratio of the molecular mass (Mw) as calculated from the experimental value and the theoretically expected molecular mass of a monomer. These results show that the CDHC purified in the absence of its accessory subunits forms either monomers or high oligomers and aggregates. In contrast to CDHC alone, the dynein complex existed as a single species under these conditions, consistent with containing a heavy-chain dimer.
Fig. S5. EM images of different negatively stained dynein subcomplexes. Galleries of single particles of the entire dynein complex and CDHC-IC-LIC, CDHC-LIC, and CDHC-IC preparations. Each image shows an area of 80 × 80 nm, except the larger images of heavy chain (HC)-IC aggregates that show 160 × 160 nm area. These results confirmed that CDHC-IC is mostly aggregated (containing only few dimers) and showed that the combinations CDHC-IC-LIC and CDHC-LIC formed mostly complexes containing dimers of the heavy chain with the tail sometimes appearing less compact.

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Fig. S6. Purification and hydrodynamic properties of the dynein heavy chain in combination with different sets of accessory subunits. This figure supplements Table 1. (A) SYPRO Ruby-stained SDS gel of the dynein heavy chain copurified with different subunit combinations. (B) Analytical ultracentrifugation: sedimentation coefficient distributions of the entire dynein complex and the combinations CDHC-IC-LIC and CDHC-LIC. The lines are Gaussian fits to the data (blue circles), with the center of the main peaks being indicated in the figure. For comparison, an overlay of the three normalized fits is shown (Lower Right). These results show that the s values of CDHC-IC-LIC and CDHC-LIC are similar to the s value of the entire dynein complex but are significantly lower. Given that the molecular mass of CDHC-IC-LIC is very similar to that of the complete complex, the lower s value indicates that the complex lacking the light chains is more elongated than the complete complex, suggesting that the tail is less compact than in the complete complex.
Fig. 57. Single-molecule TIRF microscopy data. (A) Single-molecule GFP fluorescence intensity distributions of Drosophila kinesin-1 constructs [monomeric Kin340-mGFP (light green circles) and dimeric Kin401-mGFP (cyan hexagons)] and dynein constructs [mGFP-Dyn380kD (green circles), mGFP-cc-Dyn380kD (blue diamonds), mGFP-dynein complex (red stars), and mGFP-CDHC (brown open circles)] presented as relative frequency. These data demonstrate that the dynein complex forms mostly dimers under single-molecule imaging conditions, similar to the artificially dimerized dynein motor domains (cc-Dyn380kD) and the kinesin-1 dimer (Kin401), in contrast to the monomeric kinesin (Kin340) and dynein motor domains (Dyn380kD). The dynein heavy chain alone forms a heterogeneous mixture including higher aggregates. (B) Dwell-time distributions (shown as 1-cdf) of single mGFP-Dyn380kD (Upper, blue dots) and mGFP-cc-Dyn380kD (Lower, blue dots) presented as relative frequency. The dynein complex forms mostly dimers under single-molecule imaging conditions, similar to the artificially dimerized dynein motor domains (cc-Dyn380kD) and the kinesin-1 dimer (Kin401), in contrast to the monomeric kinesin (Kin340) and dynein motor domains (Dyn380kD).
Dyn380kD (Lower, blue dots) molecules in the presence of AMP-PNP and a monoexponential fit (red lines), with mean dwell times of 1.215 ± 0.001 and 3.38 ± 0.01 s, respectively. Shown are SEM. The number of analyzed events was 1,290 and 1,071, respectively. Only interactions with clearly detectable binding and unbinding time points within the 70-s movie were analyzed. The longer dwell time of the dimeric construct in comparison with the monomeric motor domain is in agreement with the dimeric versus monomeric nature of the two constructs. (C) Single-molecule behavior of dimeric kinesin Kin401-mGFP on MTs imaged using TIRF microscopy, measured for control purposes. Exemplary kymographs showing individual Kin401-mGFP molecules interacting with MTs in the presence of 2 mM ATP or 1 mM AMP-PNP, as indicated. The concentration of Kin401-mGFP was 10 pM, and the temperature was 25 ± 1 °C. Individual Kin401-mGFP molecules moved processively along a MT in the presence of ATP. Histogram shows single Kin401-mGFP molecule velocity distribution with a Gaussian fit (red line), with a mean of 0.84 ± 0.01 μm/s. Run-length distribution and dwell-time distribution (shown as 1 – cdf) of single Kin401-mGFP molecules in the presence of ATP (blue dots) and a monoexponential fit (red line), yielding a mean run length of 1.22 ± 0.02 μm and a mean dwell time of 1.52 ± 0.02 s. Shown are SEM. The number N of analyzed events as indicated. In the presence of AMP-PNP, kinesin bound statically to MTs often for many tens of seconds.
Movie S3. MT gliding driven by artificially dimerized dynein motor domains (cc-Dyn380kD) immobilized on a Tris-Ni-NTA-PEG surface in the presence of ATP.

Movie S3

Movie S4. MT gliding driven by the dynein complex nonspecifically adsorbed on a nonfunctionalized glass in the presence of ATP.

Movie S4