37°C for 5 hours, and the peptides were separated on a LiChrospher RP-8 HPLC-Cartridge (Hewlett-Packard) with a linear acetonitrile gradient in 0.1% (v/v) TFA. Peptides were monitored at 215 and 415 nm to detect the heme-binding peptide. The peak fraction was collected, lyophilized, and resuspended in 50% (v/v) acetonitrile, 0.1% (v/v) acetate.

18. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. MS/MS analysis was performed with a micro-ion-spray ion source on a API 365 LC/MS/MS system (Perkin-Elmer) with a flow rate of 15 μl per hour. The first and third quadrupole were set to scan the mass range of 500 to 2500 *m/z*. The collision gas in the second quadrupole was nitrogen.
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23. Preparation of membranes, soluble fractions, and the periplasmic fraction and determination of protein concentration, SDS-PAGE, heme staining, protein immunoblot analysis, and visual light spectroscopy were performed as described [L. Thöny-Meyer, D. Stax, H. Hennecke, *Cell* **57**, 683 (1989); R. A. Fabianek, H. Hennecke, L. Thöny-Meyer, *J. Bacteriol.* **180**, 1947 (1998); R. Zufferey *et al.*, *J. Biol. Chem.* **273**, 6452 (1998)]. CcmE-specific antiserum directed against the synthetic peptide K^{129}HDENYTPPEVEKAME^{144} was purchased from TANA Laboratories (Houston, TX).
24. The *ccmABCDE* genes were expressed constitutively from the pACYC184-derived *tet* promoter in a Δ*ccm-ABCDEFGH* background (*8*).
25. We thank H. Troxler (Department of Pediatrics, University of Zürich) for mass spectrometry analysis, W. Staudenmann and P. James for protein sequence analysis, A. Hungerbühler for technical assistance, and M. Aebi and A. Helenius for constructive comments on the manuscript. Supported by a grant from the Swiss National Foundation for Scientific Research.

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Determinants of Kinesin Motor Polarity

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The kinesin motor protein family members move along microtubules with characteristic polarity. Chimeric motors containing the stalk and neck of the minus-end-directed motor, Ncd, fused to the motor domain of plus-end-directed kinesin were analyzed. The Ncd stalk and neck reversed kinesin motor polarity, but mutation of the Ncd neck reverted the chimeric motor to plus-end movement. Thus, residues or regions contributing to motor polarity must be present in both the Ncd neck and the kinesin motor core. The neck-motor junction was critical for Ncd minus-end movement; attachment of the neck to the stalk may also play a role.

Most kinesins (*1*) move toward the unstable plus ends of their filamentous tracks, the microtubules, but some move toward the more stable minus ends. What is the molecular basis of directionality of plus- and minus-end kinesin movement? Chimeric motor proteins consisting of the Ncd motor domain, the region of the protein that binds nucleotide and microtubules, joined to the neck and stalk of kinesin heavy chain (KHC) move toward microtubule plus ends, reversing the polarity of Ncd (*2, 3*). This finding raises several important questions about the kinesin motors. Foremost, can the Ncd stalk and neck change the direction of KHC movement? What are the determinants of motor directionality? Residues or regions of the protein must exist that contribute to or define the direction of motor movement. We examined the basis of kinesin motor polarity by constructing chimeric motors consisting of the Ncd stalk and neck joined to a KHC motor domain. The chimeric proteins were expressed in bacteria and assayed in vitro for directionality of movement on microtubules.

The design of the chimeric NcdKHC proteins analyzed in this study was guided by the crystal structures of the Ncd and KHC motor domains (*4*). The motor core (*3, 5*) refers to the region of the motor domain conserved between Ncd and KHC, extending from the start of strand β1 at the NH_2-terminus to the end of helix α6 at the COOH-terminus (*4*) (Fig. 1). The neck of Ncd (residues 329 to 348) is defined as the region between the end of the predicted α-helical coiled-coil stalk (*6*) and the start of the first conserved structural element, strand β1, of the Ncd motor core (Fig. 1) (*5*). The neck of *Drosophila* KHC (residues 327 to 337) is defined as the region between the end of the last conserved structural element, helix α6, of the KHC motor core and the beginning of the predicted coiled-coil stalk (Fig. 1) (*5*). This definition of the KHC neck differs from those of others (*7, 8*), but it provides a working definition that can be tested experimentally.

We constructed six chimeric proteins for analysis, NcdKHC1–6 (Fig. 1) (*9, 10*) and tested lysates containing the induced chimeric proteins in microtubule gliding assays for their ability to bind microtubules to the cover slip surface and support microtubule gliding (*11*). All the chimeric proteins except NcdKHC3 showed this capability. NcdKHC3 bundled microtubules in solution but did not bind microtubules to the glass surface, which indicates that the region missing from the chimeric protein, the Ncd COOH-terminus, was needed for this function. The Ncd COOH-terminus was thus included in the other chimeric constructs analyzed in this study, except NcdKHC6, in which the Ncd COOH-terminus was replaced by the KHC neck.

Polarity assays to determine directionality of motor movement on microtubules were carried out using axoneme-microtubule complexes consisting of a microtubule with a curved axoneme fragment at the minus end (*12*). NcdKHC1 showed minus-end-directed movement ($n = 42$) (Fig. 2) with a velocity of 1.4 ± 0.18 μm/min (mean ± SE; $n = 26$) (Table 1). Controls with the same preparations of axoneme-microtubule complexes showed only minus-end movement for Ncd (GST-MC1) (*13*) and plus-end movement for kinesin. Thus, the Ncd stalk and neck reversed the polarity of movement of KHC. The gliding velocity of NcdKHC1 was about 10-fold slower than Ncd (Table 1).

In contrast to NcdKHC1, NcdKHC2 in polarity assays moved only toward microtubule plus ends ($n = 10$) with a velocity of 0.23 ± 0.04 μm/min (mean ± SE; $n = 10$) (Table 1). Changing three amino acids in the neck of NcdKHC1 thus reversed the minus-end directionality of the motor and caused the mutant motor to move with the same polarity as KHC. A control with the same preparation of axoneme-microtubule complex showed only minus-end movement of Ncd (GST-MC1). The gliding velocity of NcdKHC2 was slow, >100-fold slower than kinesin and about 6-fold slower than NcdKHC1 (Table 1).

Like NcdKHC1, NcdKHC4 showed minus-end movement on microtubules ($n = 23$) but with a velocity of 0.57 ± 0.08 μm/min (mean ± SE; $n = 11$) (Table 1). NcdKHC4 differs from NcdKHC1 by only a single amino acid residue, Leu^{345} to Ala (L345A). The L345A mutation in the Ncd neck reduced motor velocity two- to threefold compared with NcdKHC1, but it did not alter the ability of the Ncd stalk and neck to reverse the polarity of KHC movement. In contrast to NcdKHC4, NcdKHC5 moved toward microtubule plus ends [$n = 22$; velocity = 0.23 ± 0.04 μm/min (mean ± SE; $n = 9$) (Table 1)]. The two-residue shift alone at the neck-motor junction of NcdKHC5 thus reverted the minus-end movement of NcdKHC1 to plus-end movement.

Polarity assays of NcdKHC6 showed minus-end movement of the chimeric motor on

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microtubules (n = 12) with a velocity of 1.4 ± 0.17 μm/min (mean ± SE; n = 11) (Table 1). Replacing the Ncd COOH-terminus with the *Drosophila* KHC neck thus reduced motor velocity about 10-fold compared with Ncd (Table 1), but it did not alter motor polarity.

The Ncd stalk and neck thus reversed the polarity of KHC movement, but mutating the Ncd neck reverted this effect, yielding a plus-end motor. The amino acids that were mutated lie near the neck-motor junction, within four residues of the start of strand β1 in the motor core (Fig. 1). Of these residues, L345 lies in a heptad repeat with hydrophobic amino acids in positions 1 and 4, characteristic of α helices and α-helical coiled coils, that extends through the Ncd neck to the start of strand β1. This heptad repeat is not part of the predicted coiled coil of the stalk but is present in the region of Ncd that we define as the neck (*5*). The L345A mutation alone in NcdKHC4 reduced motor velocity two- to threefold but did not revert the motor to plus-end movement. Thus the heptad repeat of the Ncd neck may be important for Ncd neck function as it affects motor velocity, but a change of L345A did not revert the polarity of motor movement.

The two-residue shift at the neck-motor junction of NcdKHC5 resulted in slow plus-end movement instead of the minus-end movement of NcdKHC1 and NcdKHC4. Thus the neck-motor junction is critically important for the minus-end movement of NcdKHC1 and NcdKHC4, and the neck residues immediately adjacent to the neck-motor junction are required for the minus-end movement. The simplest interpretation of the plus-end polarity of NcdKHC2 and NcdKHC5 is that the mutational changes in the Ncd neck prevent the neck from functioning properly, permitting expression of plus-end polarity determinants present in the KHC motor core. These inferred plus-end determinants result in very slow movement of the NcdKHC2 and NcdKHC5 motors on microtubules.

Replacing the COOH-terminus of Ncd, the 37 amino acids that extend beyond the motor core, with the KHC neck in NcdKHC6 reduced motor velocity about 10-fold compared with Ncd, but motor polarity was not reversed. NcdKHC6 resembles ncd-Nkin (*2*) (Fig. 1), the chimera of Ncd and *Neurospora* KHC, in that both hybrid proteins contain the Ncd motor flanked to either side by the Ncd and KHC necks. Both necks may not be complete, however, as ncd-Nkin lacks four

Table 1. Polarity of chimeric motor movement on microtubules. Axoneme-microtubule complexes (Ax-MTs) gliding on motor proteins bound to cover slips were imaged by VE-DIC microscopy and scored for polarity of motor movement. Plus-end movement is axoneme leading and minus-end movement is microtubule leading. Velocities were determined by tracking complexes from videotapes. NcdKHC1–6 (Fig. 1) (*9*) and MC1 (*13*) were GST fusion proteins and kinesin was from bovine brain (Cytoskeleton, Denver, Colorado). NcdKHC3 bundled microtubules in solution but did not bind microtubules to the cover slip surface. ND, not determined.

Motor protein	No. of Ax-MTs		Velocity (μm/min)
	Plus-end movement	Minus-end movement	
NcdKHC1	0	42	1.4 ± 0.18 (n = 26)
NcdKHC2	10	0	0.23 ± 0.04 (n = 10)
NcdKHC3	ND	ND	ND
NcdKHC4	0	23	0.57 ± 0.08 (n = 11)
NcdKHC5	22	0	0.23 ± 0.04 (n = 9)
NcdKHC6	0	12	1.4 ± 0.17 (n = 11)
Ncd (MC1)	0	177	15.2 ± 0.3 (n = 52)
Kinesin	60	0	42.7 ± 2.1 (n = 25)

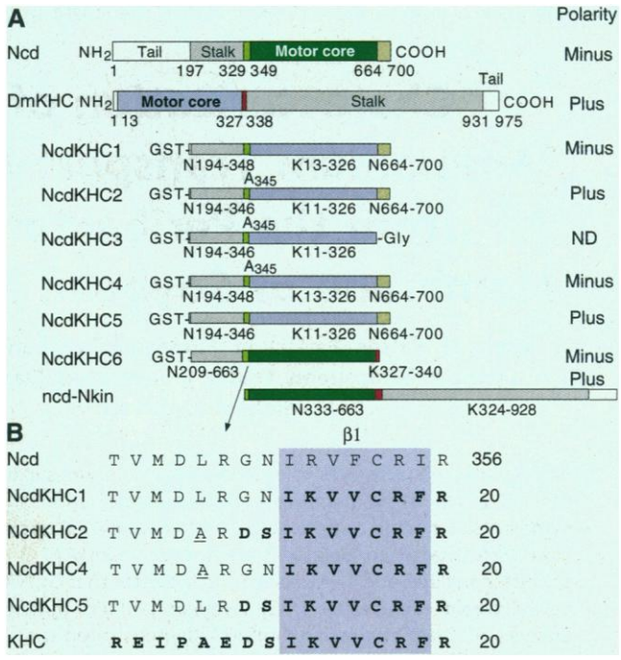

Fig. 1. Native and chimeric kinesin motor proteins. (**A**) Ncd and *Drosophila melanogaster* KHC (DmKHC) consist of a conserved motor core (Ncd, dark green; KHC, blue), stalk (gray), and tail (white). The Ncd neck is defined as residues 329 to 348 (pale green) and the DmKHC neck is defined as residues 327 to 337 (red) (see text). NcdKHC1 consists of GST fused to Ncd residues 194 to 348, followed by KHC residues 13 to 326 and Ncd residues 664 to 700 (brown). NcdKHC2–5 are mutants of NcdKHC1. NcdKHC2 contains a mutation in the Ncd neck, L345A, and a two-residue shift in the neck-motor junction. NcdKHC3 is the same as NcdKHC2, but the Ncd COOH-terminus is deleted and a Gly is added. NcdKHC4 has the L345A mutation of NcdKHC2 but not the two-residue shift, and NcdKHC5 has the two-residue shift but not the L345A mutation. NcdKHC6 consists of the Ncd stalk, neck, and motor (residues 209 to 663) followed by the *Drosophila* KHC neck (residues 327 to 340). The ncd-Nkin hybrid (*2*) consists of the Ncd neck and motor (residues 333 to 663) joined to the *Neurospora* KHC, Nkin, and neck and stalk (residues 324 to 928). ncd-Nkin residues are renumbered from (*2*) to fit our definition of the Nkin neck (residues 324 to 342) and Ncd motor core (*5*). NcdKHC3 did not bind microtubules to the cover slip in motility assays. ND, not determined. (**B**) Neck-motor junction sequences. Sequences at the neck-motor junction of NcdKHC1 and three mutant NcdKHC motors are shown together with Ncd and KHC (boldface). Residues that form strand β1 of the motor core are shaded blue. NcdKHC2 and NcdKHC4 contain residue L345A, and the NcdKHC2 and NcdKHC5 neck-motor junctions are shifted by two residues compared with NcdKHC1. NcdKHC2 differs from NcdKHC1 by three residues, NcdKHC4 by a single residue (L345A), and NcdKHC5 by two residues, all within the Ncd neck. Numbers shown for the NcdKHC proteins refer to DmKHC residues.

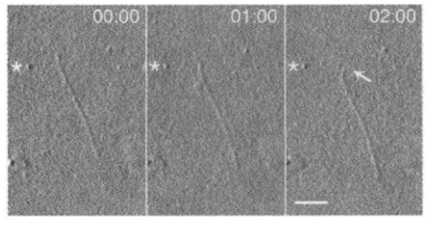

Fig. 2. NcdKHC1 moves toward microtubule minus ends. An axoneme-microtubule complex gliding on NcdKHC1 bound to a cover slip is moving with the microtubule end of the complex leading, indicating that the chimeric motor moves toward the microtubule minus end. The gliding velocity was 1.01 μm/min. (**Right**) The curved axoneme fragment is indicated by an arrow. An asterisk is adjacent to a fixed point on the surface. Time is in minutes and seconds. Bar = 3 μm.

residues of the Ncd neck by our definition (*5*) and NcdKHC6 lacks four residues of the KHC neck according to the dimer KHC crystal structure in which the coiled coil begins four residues COOH-terminal to the start of the heptad repeats (*8*). The hybrid proteins also differ in that NcdKHC6 has the Ncd stalk at the NH_2-terminus of the motor, joined to the Ncd neck, whereas ncd-Nkin has the KHC stalk at the COOH-terminus, joined to the KHC neck (Fig. 1). Strikingly, NcdKHC6 is a minus-end motor and ncd-Nkin is a plus-end motor (*2*). Thus an intact neck, or the attachment of the neck to the stalk, may be important for motor polarity rather than simply the neck and neck-motor junction.

The NcdKHC1 motor was slow compared with Ncd (Table 1), suggesting that the neck-motor junction, or another aspect of the coupling of the motor to the stalk and neck, is imperfect. NcdKHC2 and NcdKHC5 were even slower than NcdKHC1, suggesting that the Ncd neck or neck-motor junction may affect motor velocity. The neck of myosin, a molecular motor with structural similarity to kinesin and Ncd (*4*), has been shown to contribute to motor velocity (*14*), although the effects of the myosin and Ncd necks on motor velocity may differ. Our results indicate that polarity determinants, residues or regions that contribute to the direction of motor movement, are present in the Ncd neck and the KHC motor core. Neck residues immediately adjacent to the conserved motor core are critical for Ncd minus-end movement and attachment to the stalk may also be important.

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9. NcdKHC1 consists of glutathione S-transferase (GST) fused to the Ncd stalk and neck followed by the *Drosophila* KHC motor core and the Ncd COOH-terminus [residues 664 to 700 that extend beyond the conserved motor core and may interact with the Ncd neck (*4*)]. NcdKHC2–5 are mutants of NcdKHC1 (Fig. 1). NcdKHC6 consists of the Ncd stalk, neck, and motor with the Ncd COOH-terminus replaced by the *Drosophila* KHC neck.
10. The *pGEX/ncdkhc1–5* chimeric plasmids were constructed from a plasmid coding for GST fused to Ncd residues 194 to 346 [S. A. Endow, S. Henikoff, L. Soler-Niedziela, *Nature* **345**, 81 (1990)], followed by *Drosophila* KHC residues 11 to 340 [J. T. Yang, R. A. Laymon, L. S. B. Goldstein, *Cell* **56**, 879 (1989)]. The plasmid was constructed by using the polymerase chain reaction (PCR) and wild-type *ncd* and *khc* plasmids [H. Song and S. A. Endow, *Biochemistry* **35**, 11203 (1996)]. A Bam HI site was created to ligate *ncd* to *pGEX-2T* [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)] and a Bss HII site was created for the junction with *khc*, chosen because it minimally altered the Ncd neck according to the computer program COILS (*6*), changing L345A. The *khc* primers contained a Bss HII site, sequences coding for residues 338 to 340 missing from the *khc* plasmid, and an Eco RI site for ligation to *pGEX-2T*. *pGEX/ncdkhc2* and *-3* were made by replacing the Nsi I–Eco RI fragment of the plasmid with overlap extension PCR fragments [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)] to substitute Ncd residues 664 to 700 for KHC residues 327 to 340 or to delete KHC after residue 326 and add a glycine. *pGEX/ncdkhc1*, *-4*, and *-5* were made from *pGEX/ncdkhc2* by replacing the Sph I–Nsi I fragment with overlap PCR fragments that repaired the L345A mutation in the Ncd neck or shifted the neck-motor junction by two residues or both. *pGEX/ncdkhc6* was made from *pGEX/MC1* (*13*) and wild-type *khc* by replacing the *pGEX/MC1* Bst EII–Eco RI fragment with an overlap PCR fragment coding for Ncd to residue 663, followed by KHC residues 327 to 340. All plasmids were sequenced to confirm the intended changes and exclude PCR mutations.
11. The chimeric plasmids were transformed into *BL21(DE3)pLysS* cells for protein expression [F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *Methods Enzymol.* **185**, 60 (1990)]. Cells were grown in M9ZB to an OD_{550} of 0.65 to 1 and induced at 21° to 22°C. Induction times, usually 5 to 6 hours, were optimized by analysis on polyacrylamide gels. The molecular weight of the major induced protein band in each case corresponded to the predicted molecular weight of the hybrid protein. Lysates for motility assays were prepared as described in (*15*). Microtubule gliding assays were carried out by video-enhanced differential interference contrast (VE-DIC) microscopy [R. A. Walker *et al.*, *J. Cell Biol.* **107**, 1437 (1988)]. Samples of cell lysates used in motility experiments were analyzed on immunoblots. Proteins were reacted with antibody to HIPER, directed against a highly conserved sequence motif in the kinesin motor domain [K. E. Sawin, T. J. Mitchison, L. G. Wordeman, *J. Cell Sci.* **101**, 303 (1992)] (gift of K. Sawin, ICRF, London) or an antibody to Ncd COOH-terminus residues 670 to 682 [M. Hatsumi and S. A. Endow, *J. Cell Sci.* **103**, 1013 (1992)] and detected with an alkaline phosphatase system. A single major band of the predicted molecular weight was observed in each case, indicating that the chimeric proteins in the lysates used for the motility assays were intact.
12. Axoneme-microtubule complexes were prepared as described in (*15*). To determine gliding velocities, we tracked axoneme-microtubule complexes with a custom tracking program (a gift of N. Gliksman and T. Salmon, University of North Carolina, Chapel Hill, NC).
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Characterization of an Ammonium Transport Protein from the Peribacteroid Membrane of Soybean Nodules

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Nitrogen-fixing bacteroids in legume root nodules are surrounded by the plant-derived peribacteroid membrane, which controls nutrient transfer between the symbionts. A nodule complementary DNA (*GmSAT1*) encoding an ammonium transporter has been isolated from soybean. *GmSAT1* is preferentially transcribed in nodules and immunoblotting indicates that GmSAT1 is located on the peribacteroid membrane. [^{14}C]methylammonium uptake and patch-clamp analysis of yeast expressing GmSAT1 demonstrated that it shares properties with a soybean peribacteroid membrane NH_4^+ channel described elsewhere. GmSAT1 is likely to be involved in the transfer of fixed nitrogen from the bacteroid to the host.

Many legumes form a symbiotic relationship with N_2-fixing bacteria called rhizobia, which enables the plants to grow on soils depleted of combined nitrogen. Differentiated rhizobia, called bacteroids, are housed in specialized organs (nodules) on the legume roots. Within the infected cells of nodules, N_2-fixing bacteroids are enclosed by the plant-derived peribacteroid membrane (PBM), which segregates the bacteroids from the plant cytosol and controls nutrient exchange between the symbionts. The organelle-like structure consisting of PBM, bacteroid, and the intervening peribacteroid space (PBS) is known as the symbiosome (*1*). In soybean, NH_4^+ is probably the main form of nitrogen exported from the symbiosome (*2*) and analysis of PBM isolated from soybean and pea has identified a monovalent cation channel with