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The *Neurospora* Organelle Motor: A Distant Relative of Conventional Kinesin with Unconventional Properties

Gero Steinberg and Manfred Schliwa*

Institute for Cell Biology, University of Munich, Schillerstraβe 42, 80336 Munich, Germany

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The "conventional" kinesins comprise a conserved family of molecular motors for organelle transport that have been identified in various animal species. Organelle motors from other phyla have not yet been analyzed at the molecular level. Here we report the identification, biochemical and immunological characterization, and molecular cloning of a cytoplasmic motor in a "lower" eukaryote, the Ascomycete fungus *Neurospora crassa*. This motor, termed Nkin (for Neurospora kinesin), exhibits several unique structural and functional features, including a high rate of microtubule transport, a lack of copurifying light chains, a second P-loop motif, and an overall sequence organization reminiscent of a kinesin-like protein. However, a greater than average sequence homology in the motor domain and the presence of a highly conserved region in the C-terminus identify Nkin as a distant relative of the family of conventional kinesins. A molecular phylogenetic analysis suggests Nkin to have diverged early in the evolution of this family of motors. The discovery of Nkin may help identify domains important for specific biological functions in conventional kinesins.

INTRODUCTION

The kinesin superfamily comprises a growing number of microtubule motor proteins that can be distinguished from one another on the basis of sequence similarity, domain structure, and physiological function. Within this broad scheme, a family of conventional kinesins (also referred to as true, standard, or classical kinesins) is usually distinguished from several families of kinesin-like proteins (for reviews, see Goldstein, 1993; Bloom and Endow, 1994). The unifying feature of the members of this superfamily is the presence of a motor domain of about 350 amino acids that possesses binding sites for both ATP and microtubules and that is believed to generate the force for motility. The motor domain of the kinesin-like molecules typically is 30–40% identical to the motor domain of conventional kinesins (Endow and Hatsumi, 1991; Goldstein, 1993; Bloom and Endow, 1994). Among the kinesins whose motor activity could directly be tested in a microtubule-gliding assay (Paschal and Vallee, 1993), most display plus end-directed movement, but two motors with minus end-directed movement, ncd and KAR3, are also known (Walker *et al.*, 1990; Endow *et al.*, 1994). Functional specificity is conferred to each member of the kinesin superfamily by the nonmotor domain, in particular, a presumably globular tail region present at the end of the polypeptide chain opposite to the motor domain.

Among the families and subfamilies of kinesins identified so far, that of the conventional kinesins is the most coherent and best characterized. Motors of this family have been isolated from various animal tissues and organisms, including squid axons (Brady, 1985; Vale *et al.*, 1985), bovine brain (Kuznetsov and Gelfand, 1986; Bloom *et al.*, 1988), sea urchin eggs (Scholey *et al.*, 1985), *Drosophila* (Saxton *et al.*, 1988), and bovine adrenal medulla (Murofushi, 1988). Conventional kinesin is a tetrameric complex of two heavy chains of 120–130 kDa possessing an N-terminal motor domain and two light chains of 60–70 kDa associated with the heavy chains in their tail domain (Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988; Hirokawa *et al.*,

* Corresponding author.
Abbreviations follow the standard nomenclature for kinesins (see Bloom and Endow, 1994).

1989). The members of this family have been strongly implicated in the movement of various membrane-bounded organelles (e.g. Dabora and Sheetz, 1988; Schroer *et al.*, 1988; Hirokawa *et al.*, 1991; Leopold *et al.*, 1992). Kinesin colocalizes with organelles (Hollenbeck, 1989; Pfister *et al.*, 1989; Wright *et al.*, 1991; Toyoshima *et al.*, 1992), and antibodies against kinesin inhibit organelle movements in melanophores (Rodionov *et al.*, 1991), squid axoplasm (Brady *et al.*, 1990), and macrophages (Hollenbeck and Swanson, 1990). Based on this and additional evidence (reviewed in Bloom and Endow, 1994), conventional kinesins are considered motor proteins for the transport of membrane-bounded organelles toward microtubule plus-ends.

Although the family of conventional kinesins as we know it to date presents itself as an homogeneous protein family, its members cover a rather limited phylogenetic spectrum (animals only, ranging from Nematodes to Vertebrates). Very little is known about related molecules of the kingdoms of plants, fungi, and protoctista. Motors with kinesin-like properties have been partially purified from *Acanthamoeba* (Kachar *et al.*, 1987) and *Dictyostelium* (McCaffrey and Vale, 1989), but their molecular identity is uncertain. In addition, organelle motors unrelated to conventional kinesins seem to exist (Aizawa *et al.*, 1992; Nangaku *et al.*, 1994).

Here we report on the isolation, initial biochemical and immunological characterization, and molecular cloning of a member of the kinesin family from *Neurospora crassa*, the first to be isolated in a native state from a representative of the kingdom of fungi. By a number of criteria, it is an organelle motor, and it represents the major microtubule motor present in the cytoplasm. This motor, which we termed Nkin (for Neurospora kinesin), appears to be a distant relative of the conventional kinesins. The existence of Nkin may change the way we view the family of conventional kinesins, and offers novel opportunities for the study of kinesin organelle motors.

MATERIALS AND METHODS

Strains and Growth Conditions

N. crassa wild-type 74A was grown in Vogel's minimal medium at 25°C under continuous aeration and illumination with white light (Sebald *et al.*, 1979). The cell wall-less mutants fz;sg;os-1, number FGSC 1118, and FGSC 4761 (referred to as SLIME; Emerson, 1963) were cultured on nutrient-enriched Vogel's medium agar plates. For biochemical experiments, liquid cultures were inoculated with SLIME and the cells were grown under gentle shaking and illumination at 30°C for 3–7 days (according to the method of Smith and Scarborough, 1984).

Isolation of a Native Cytoplasmic Motor Enzyme

N. crassa wild-type 74A was grown for 16 h, harvested, and ground with quartz-sand in the presence of AP100 buffer (100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, pH 6.9, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml Tosyl-arginie-methyl esters, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin). All steps were performed at 4°C. After a low speed centrifugation to remove the sand and cell debris at 10000 × *g* for 20 min, a high speed supernatant (S2) depleted of organelles was prepared (120,000 × *g*, 1 h). Microtubules prepared from PC-purified porcine brain tubulin (modified from the method of Mandelkow *et al.*, 1985) were stabilized with taxol and added to S2 at a concentration of 0.8 mg/ml. Additional taxol (7 μM) as well as apyrase (5 U/ml; Grade VIII, Sigma, St. Louis, MO) were added to the S2 and the mixture was incubated on ice for 1 h. In other experiments 2–4 mM AMP-PNP or 50 U/ml hexokinase plus 10 mM glucose were used in place of apyrase. The microtubules were sedimentated (100,000 × *g*, 30 min), carefully resuspended in AP100/50 mM KCl, and centrifuged through a 10% sucrose cushion in AP100 (90000 × *g*, 30 min). The resulting microtubule pellet (P4) was resuspended in AP100 with 5–10 mM ATP and placed on ice for 10 min. After centrifugation (100,000 × *g*, 30 min) the resulting supernatant (S5) was tested in a gliding assay (see below). The S5 was stored on ice and normally stayed active for several days. For additional purification the motor activity was fractionated on linear 5–20% sucrose gradients (5 ml) in AP100 and centrifuged at 130,000 × *g* in a Beckmann SW 50.1 rotor for 13 h. Fractions of 100–300 μl were collected from the bottom and checked for motor activity in gliding assays. For gel filtration a superose-6 column was used in fast performance liquid chromatography (FPLC) (Pharmacia, Piscataway, NJ). The column was equilibrated with AP100/150 mM NaCl/1 mM dithiothreitol/0.1 mM ATP. Fractions (400–500 μl) were collected. Fractions shown to be active in a gliding assay were usually concentrated for further use (microcon tubes, Amicon, Beverly, MA). Bovine brain kinesin was isolated according to the method of Kuznetsov and Gelfand (1986). Protein concentrations were determined according to the method of Bradford (1976).

Motility Assay

For a gliding assay, 4 μl of S5 or motor-containing fractions were placed on no. 1 coverslip for 1–5 min, and taxol-stabilized microtubules prepared from purified tubulin (~0.04 μg/ml; see above) and MgNTP (final concentration ~4 mM) were added. The coverslip was sealed with a mixture of Vaseline, lanolin, and paraffin (1:1:1, VALAP). Motility was monitored by video-enhanced DIC microscopy on a ZEISS axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Hamamatsu DVS-1000 Image Processing System and a Panasonic AG 6720 video tape recorder.

To determine the polarity of movement, *Chlamydomonas reinhardii* axonemes were isolated as described by Witman (1986) and depleted of endogenous motors by salt-extraction (King *et al.*, 1986). The polarity of motility was judged by the direction of movement of the axonemes on motor-molecules adsorbed onto a glass coverslip. In addition, polarity of movement was determined by placing latex-beads (0.5 μm in diameter) coated with motor on axonemes using a lasertrap (Ashkin *et al.*, 1990). The beads were incubated with purified motor (20–60 μg/ml) and 1 mg/ml casein for 10–15 min and rinsed by pelleting and resuspension in AP 100 buffer.

ATPase-Assay

The specific ATPase activity of purified motor was measured with the malachite-green method (modified from Lill *et al.*, 1990). Column-purified peak fractions of motor were concentrated to ~100 μg/ml using microcon tubes (Amicon) and incubated in a reaction mixture with MgATP (final concentration 3 mM) and either taxol-stabilized microtubules or AP100 buffer alone. To avoid phosphate contamination the microtubules were polymerized at high concentration (>10 mg/ml tubulin) with just 0.1 mM GTP. Control experiments were run with the same ingredients minus motor. The reaction mix was incubated for 10 min at 30°C and the reaction was

stopped by the addition of highly acidic MGR (0.34% malachite green/1% ammonium molybdate/1 M HCl/0.1% Triton-X 100). The amount of released phosphate was determined by comparison with a calibration curve prepared with $NaHPO_4$.

Antibody Preparation and Affinity Purification

A female New Zealand White rabbit was immunized with S5 containing the *Neurospora* motor (Eurogentec, Seraing, Belgium). For inital injections, 500 μl of S5 (30–50 μg of motor) were emulsified 1:1 in complete Freund's adjuvant and injected at multiple sites. Three further boosts were performed 15, 30, and 60 days after the first immunization, using incomplete Freund's adjuvant. The rabbit was bled 10–14 days after each boost.

Antibodies were purified essentially as described by Olmsted (1986). Peak fractions from the gel filtration column were separated by SDS-PAGE (Laemmli, 1970) in 4.8% gels to increase the separation between the 105-kDa and the 108-kDa bands, electroblotted onto nitrocellulose membranes (Towbin *et al.*, 1979), and stained with Ponceau red. Blot strips of the 105-kDa or the 108-kDa band were cut out, leaving a gap of 1–2 mm between them. The cut-out strips were blocked with TBST (20 mM Tris, pH 7.2, 150 mM NaCl, 0.05% Tween-20/0, 1% NaN_3) containing 5% (w/v) milk powder for 45 min at room temperature, washed three times in TBST, and incubated for several hours in an aliquot of serum. The antibodies were eluted by vortexing the blot strips in 200 μl of 100 mM glycine/HCl (pH 2.5) for 1 min, followed immediately by neutralization with 1 M Tris-base. The purified antibodies were stored at −80°C. They could be used at a dilution of 1:500 to 1:1000.

Western Blot Analysis and Indirect Immunofluorescence

Proteins were separated by SDS-PAGE on 7% gels and electroblotted as described above. The membranes were incubated (45 min, 20°C) with affinity-purified antibodies, rinsed with TBST, and incubated with the secondary peroxidase-conjugated antibodies (goat anti-rabbit IgG, at 1:1500 dilution) for 45 min at room temperature. Following three rinses in TBS the blot membranes were reacted with 4-chloronaphtol (10 μM) in TBS/17% MetOH/0,05% H_2O_2 or with ECL (Amersham, Buckinghamshire, UK).

For indirect immunofluorescence, cell fragments of SLIME were prepared as described (Steinberg and Schliwa, 1993). They were fixed with 0.5% glutaraldehyde/0.2% Triton X-100 in PHEM buffer (60 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid/20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid/10 mM EGTA/2 mM $MgCl_2$; Schliwa and van Blerkom, 1981) for 10 min, rinsed three times with PHEM, and treated with borohydride (2 mg/ml in PHEM). Formaldehyde and methanol fixation were also used. The specimen was incubated with affinity-purified anti-105-/108-kDa antibodies (1:1000 in PHEM) or the monoclonal anti-tubulin antibody WA3 (1:1000 in PHEM; prepared and provided by Dr. U. Euteneuer) for 45 min at room temperature. After three rinses with PHEM (5 min each), the specimen was incubated for 45 min with fluorochrome-conjugated secondary antibodies (CY3-conjugated goat anti-rabbit IgG, Dianova at 1:1000, and FITC-conjugated mouse IgG at 1:1000, both in PHEM).

Molecular Cloning Procedures

The affinity-purified anti-105-/108-kDa antibodies were used to screen a λgt-11 cDNA libary of *N. crassa* (Schneider *et al.*, 1990). One alone was identified and subcloned in pUC19 according to standard procedures (Sambrook *et al.*, 1989). Sequence analysis using [α-^{35}S]dATP and the Sequenase kit 2.0 (United States Biochemical, Cleveland, OH) revealed the clone to contain ~2400 bp of NKIN-DNA. Rescreening of >2 million phages from the cDNA-libary with a specific radioactive probe derived from the N-terminal portion of the cDNA did not yield a complete clone. Peptide sequencing was done by U. Plessmann and Dr. K. Weber in the Max Plank Institute of Biophysical Chemistry (Göttingen, Germany) as described (Ersfeld *et al.*, 1993).

5′ RACE

The missing 5′-end of the cDNA (~820 bp) was obtained by rapid amplification of cDNA ends (RACE; Frohman *et al.*, 1988). *N. crassa* wild-type cells were grown for 16 h and the total RNA was isolated according to the method of Chambers *et al.* (1986). The poly(A) mRNA was purified using an oligotex-dt poly(A) mRNA purification kit (QIAGEN, Chatsworth, CA) and first-strand cDNA synthesis was performed using a single-strand cDNA synthesis kit and an Nkin-specific primer ($_{1068}$ATCGTTGTAACTACTGGG$_{1051}$). After a poly(A) tail was added to the 3′ end of the single strand cDNA using terminal transferase (Boehringer, Ingelheim), the cDNA was purified with the QIAquick Spin PCR Purification Kit (QIAGEN). The following steps of 5′ RACE followed standard procedures. The RACE product was ligated in pUC18 and transformed in *Escherichia coli* SURE (Stratagene, La Jolla, CA). Three clones containing the plasmid were isolated and the DNA was sequenced on both strands. An overlap of ~120 bp between the RACE-fragment and the cDNA-clone covering the second P-loop motif ensured both parts to be specific Nkin-DNA. The C-terminal half of the cDNA clone was confirmed by sequencing three genomic clones.

Sequence Analysis

Sequence assembly and analysis were done with the GCG sequence analysis software package (version 7, Genetics Computer Group 1991, University of Wisconsin, Madison, WI) and the program package PIR (Protein Identification Resource, NBRF Institute, National Biochemical Research Foundation, Washington, DC). Identities were obtained from FASTA database searches (Pearson and Lipman, 1988) and similarities from sequence comparison with BESTFIT (Devereux *et al.*, 1984). Sequence alignments were performed from the program CLUSTAL V (Higgins *et al.*, 1992) and the matrix plot comparison was done using the programs COMPARE and DOTPLOT (Devereux *et al.*, 1984). The phylogenetic trees were drawn with the program DARWIN (Gonnet, 1994; Computational Biochemistry Group at ETH, Zürich, Switzerland), which is based on a maximum likelihood method and the 1% PAM-matrix of Dayhoff *et al.* (1978).

RESULTS

Purification of a Microtubule-dependent Motility Protein

Organelle transport in hyphae, protoplasts, and wall-less SLIME mutants of *N. crassa* is microtubule dependent (Steinberg and Schliwa, 1993). To isolate a candidate motor protein, we have adapted an isolation protocol commonly used in the purification of microtubule-dependent motor molecules (Vale *et al.*, 1985; Paschal *et al.*, 1987; Euteneuer *et al.*, 1988; Wagner *et al.*, 1991; Cole *et al.*, 1992) to *N. crassa*. The protocol involved the generation of a high-speed cytoplasmic extract, depletion of ATP with apyrase, cosedimention with exogenous, taxol-stabilized microtubules, and release of potential motility proteins with ATP. An important step in the protocol is the gentle grinding of hyphae with quartz-sand to break open the cell wall. The material released from microtubules with ATP (S5) supported unidirectional movement of microtu-

bules when adsorbed onto glass coverslips (see below). Besides tubulin, the two most prominent polypeptides that cosedimented with and were released from microtubules in S5 migrated at 180 kDa and 105/108 kDa in SDS-PAGE (Figure 1). Isolation protocols with hexokinase/glucose or AMP-PNP in place of apyrase yielded S5 in which the relative proportions of the 180-kDa and 105-/108-kDa polypeptides varied. Routinely, however, the purification protocol included apyrase.

To determine which of the polypeptides is responsible for the microtubule gliding activity observed in S5, the supernatant released with MgATP was subjected to sucrose density centrifugation on a 5–20% sucrose gradient (Figure 2A). Only the fractions enriched for the 105-/108-kDa doublet supported vigorous gliding of virtually all microtubules in a sample after adsorption onto glass coverslips. Further purification was achieved with gel filtration on a superose-6 FPLC column (Figure 2B). Again, microtubule motility co-fractionated with the 105-/108-kDa doublet, implicating it as the force-producing protein. Although purified 180-kDa polypeptide possessed a low level of microtubule-stimulated ATPase activity (0.025 μmol/min/mg basal activity; 0.05 μmol/min/mg in the presence of microtubules, n = 3), no microtubule movement was ever observed. After FPLC on superose-6 the 105-/108-kDa polypeptides constitute 85–90% of the total protein according to densitometry of Coomassie-stained polyacrylamide gels. Significantly, there is no evidence for light chain homologues or other co-purifying polypeptides even when the fractions containing the 105-/108-kDa doublet were run on 15% polyacrylamide gels to identify low molecular weight components and when both 7.5% and 15% gels were probed with silver staining (Figure 2C).

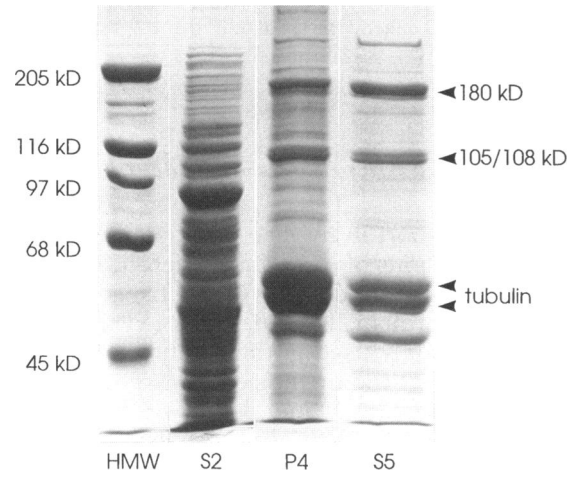

Figure 1. ATP-dependent binding to, and release from, microtubules. Coomassie blue-stained 7.5% polyacrylamide gel. Lane 1, MW markers; lane 2, cytoplasmic extract (S2); lane 3, polypeptides that bound to and co-pelleted with phosphocellulose-purified pig brain microtubules in the absence of ATP (P4); lane 4, polypeptides released from microtubules as prepared in lane 3 by 10 mM MgATP (S5). The major polypeptides released by ATP have MW of 180 kDa and 105/108 kDa.

Motility Properties of the Motor Protein

The purified *Neurospora* motor supported microtubule gliding at a rate of 2.56 ± 0.39 μm/s (n = 130) at 24°C in the presence of 4 mM MgATP. At 33°C, the rate of movement reached 4.1 μm/s. Motility could also be induced by MgGTP, MgCTP, and MgUTP at between 10 and 50% of the rate in ATP. The minimum concentration of motor that can support MgATP-dependent gliding was 30 μg/ml, which is very similar to that of other purified, native kinesins (Vale *et al.*, 1985; Cohn *et al.*, 1987). The polarity of movement was determined using flagellar axonemes isolated from *Chlamydomonas reinhardii* (Figure 3). Axonemes were observed to glide on motor-coated coverslips exclusively with the "blunt" end leading (n = 12), while latex beads (0.5-μm diameter) moved toward the splayed end of *Chlamydomonas* axonemes only. In this case the rate of movement was 1.7 ± 0.4 μm/s (n = 50). Because the splayed and blunt ends of isolated *Chlamydomonas* axonemes are a reliable marker for microtubule polarity, corresponding to the plus and minus ends, respectively, (Paschal and Vallee, 1993), the movement of the *Neurospora* motor is plus end-directed. The specific ATPase activity of superose-purified motor was determined in five separate preparations. Basal activity in the absence of microtubules ranged from 6–21 nmol/min/mg protein. The corresponding values in the presence of 1 mg/ml purified bovine brain microtubules ranged from 32–118 nmol/min/mg protein. The degree of microtubule stimulation in these five experiments ranged from 4.9x to 6.4x. While a microtubule-activated ATPase activity of 0.3 ATP/s/head, as determined here for the native molecule, appears to be too low to be compatible with a rate of microtubule motility of 2–3 μm/s, it is within the same range as that reported for other biochemically isolated kinesins (Cohn *et al.*, 1987; Bloom *et al.*, 1988; Saxton *et al.*, 1988; Wagner *et al.*, 1989).

Immunological Characteristics of the Neurospora Motor

Polyclonal antibodies were generated in rabbits by immunization with S5 and subsequent blot purification (for details, see MATERIALS AND METHODS). They recognize only the 105-/108-kDa doublet in whole cell extracts (Figure 4). Purified antibodies against either the 105-kDa or the 108-kDa antigen (see MATERIALS AND METHODS) react with both polypeptides in Western blots (Figure 4). The finding that either antibody reacts with both the 105-kDa and the 108-kDa polypeptide suggests that they represent

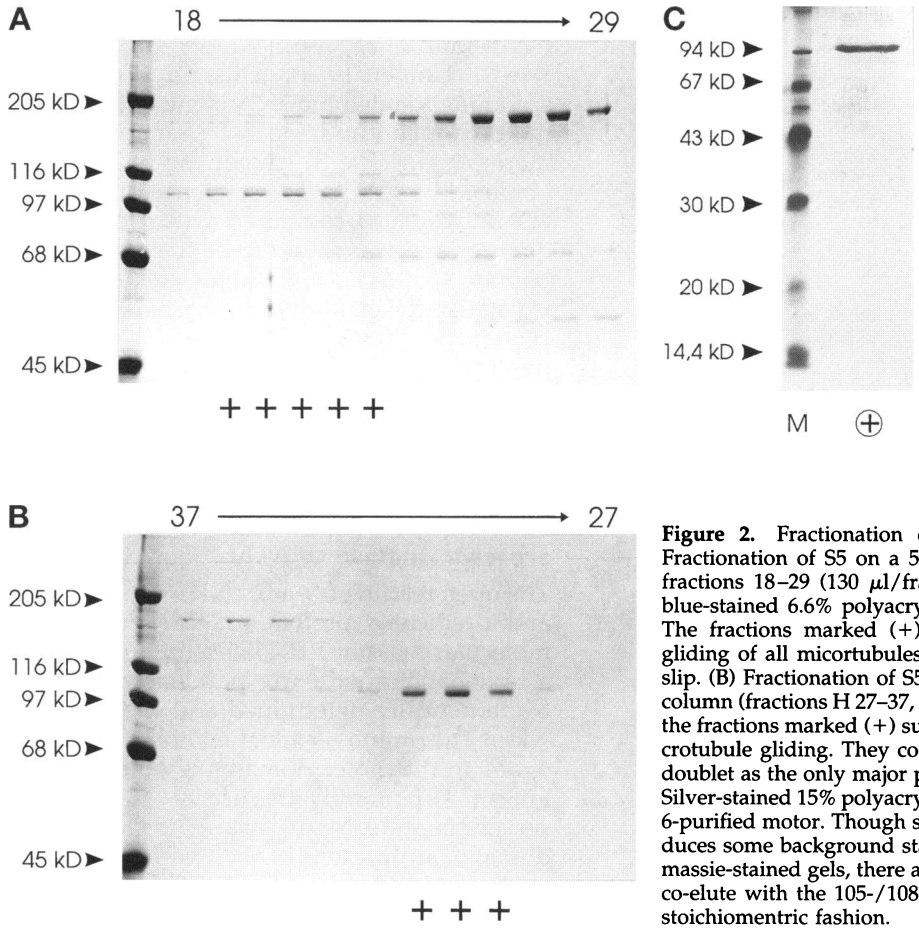

Figure 2. Fractionation of motor activity. (A) Fractionation of S5 on a 5–20% sucrose gradient; fractions 18–29 (130 μl/fraction) of a Coomassie blue-stained 6.6% polyacrylamide gel are shown. The fractions marked (+) supported prominent gliding of all micortubules attached to the coverslip. (B) Fractionation of S5 on a superose-6 FPLC column (fractions H 27–37, 400 μl/fraction). Again, the fractions marked (+) supported prominent microtubule gliding. They contain the 105-/108-kDa doublet as the only major polypeptide species. (C) Silver-stained 15% polyacrylamide gel of superose-6-purified motor. Though silver enhancement produces some background staining not seen in Coomassie-stained gels, there are no polypeptides that co-elute with the 105-/108-kDa polypeptides in a stoichiomentric fashion.

isoforms of the same protein, possibly due to post-translational modification. The proportion of the two bands varies from preparation to preparation and also differs depending on whether wild-type or SLIME cells are used.

Affinity-purified anti-105-/108-kDa antibody did not cross-react with bovine brain or sea urchin kinesin (kindly provided by J. Scholey, University of California at Davis, Davis, CA). Conversely, monoclonal antibody SUK4 (also provided by J. Scholey), which reacts with the heavy chain of conventional kinesins (Ingold *et al.*, 1988) and presumably binds within the head/stalk junction (Scholey *et al.*, 1989), does not recognize the *Neurospora* motor (Figure 4). Both the 105-kDa and 108-kDa polypeptides are, however, recognized by monoclonal antibody MMR44 (kindly provided by M. McNiven, Mayo Clinic, Rochester, MN) prepared against a peptide from the C-terminal portion of the motor domain (Marks *et al.*, 1994). In contrast to studies using other peptide antibodies (Cole *et al.*, 1992; Sawin *et al.*, 1992) where several kinesin species are recognized, no polypeptides other than the 105-/108-kDa doublet react with MMR44.

Immunolocalization of the 105-/108-kDa Antigen

When permeabilized protoplasts or the wall-less *Neurospora* mutant SLIME were used for immunofluorescence microscopy, these large, generally rounded cells are stained very brightly, and details of the localization are difficult to discern. We therefore resorted to the flat cell fragments that are left adhering to the coverslips after gentle shearing (Steinberg and Schliwa, 1993). These cell fragments still exhibit vectorial organelle transport and have already aided in the immunolocalization of microtubules. In double labeling experiments with anti-105-/108-kDa antibody and tubulin antibody, small vesicular structures frequently associated with microtubules are clearly labeled (Figures 5 and 6). Thus the antigen appears to be associated with intracellular membraneous organelles, consistent with a role of the antigen in organelle movements. Nevertheless, as in the case of other conventional kinesins purified from cells or tissues (Hollenbeck, 1989), a substantial proportion of the *Neurospora* motor appears in the soluble cytoplasmic pool even if isolations were performed under

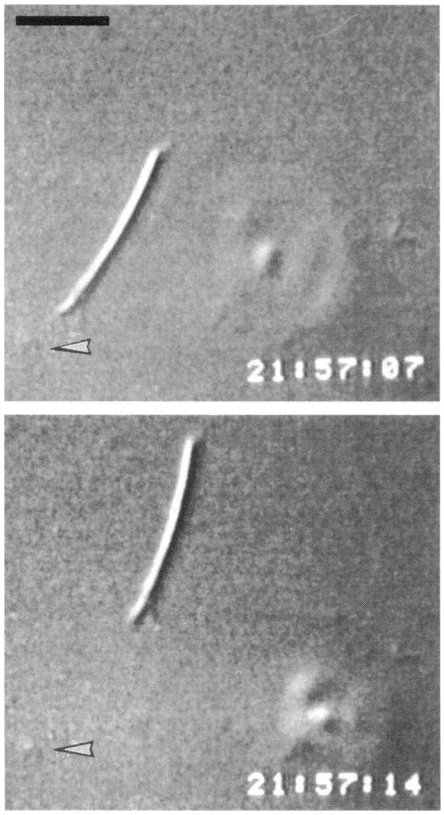

Figure 3. Gliding of a flagellar axoneme of *Chlamydomonas* on *Neurospora* motor. Gliding occurs with the blunt (minus) end leading, indicating that motor activity is directed toward the plus end. Arrowhead marks a particle attached to the coverslip. Time is shown in the lower right. Bar, 5 μm.

low-salt conditions (10 mM buffer) that minimize the dissociation of membrane-associated motor.

Cloning of the Neurospora Motor Molecule

Affinity-purified anti-105-/108-kDa antibody was used to screen an oligo dT-primed λgt11 expression library prepared from exponentially growing hyphae (Schneider *et al.*, 1990; kindly provided by Dr. H. Weiss, University of Düsseldorf, Germany). The screening yielded a cDNA clone encoding part of the motor domain and the C-terminal half of a kinesin motor, Nkin. The 5′ end of the Nkin transcript was obtained by RACE. The sequence of the 3′-end of the cDNA was also compared with cloned genomic sequences and was found to be free of error. Numerous peptide sequences were obtained from biochemically isolated and electrophoretically purified 105-/108-kDa protein (K. Weber, MPI Göttingen, Germany). These peptide sequences were found to be part of the deduced amino acid sequence of the Nkin open reading frame (boxed peptides in Figure 7), demonstrating the cloned cDNA to encode the full-length *Neurospora* motor protein.

Sequence Analysis of Nkin

The open reading frame of Nkin was 2781 bp in length and predicts a protein of 926 amino acids with a molecular mass of 102,455 Da (Figure 7). Thus there is a reasonably good correspondence between the electrophoretically determined and the predicted size of Nkin. The amino-terminal end (aa 1–337) was homologous to the motor domains of other members of the kinesin superfamily (Figure 8). All the highly conserved peptide motifs shared by all kinesin motor domains are present in their respective positions, including the P-loop motif GQTGAGKS (aa 88–95), the motif SSRSHS (aa 205–210), the motif VDLAGSE (aa 234–240), and the motif HVPYRDSKLT (aa 279–288). The rest of the molecule, starting at amino acid 338, shows very little homology to any other known kinesin, with the exception of a peptide motif near the C-terminus (discussed below).

The sequence organization of Nkin reveals several unique features. 1) In addition to the P-loop motif found in all other kinesins (aa 88–95), Nkin possesses a second motif GSEKVGKT (aa 238–245), which fits

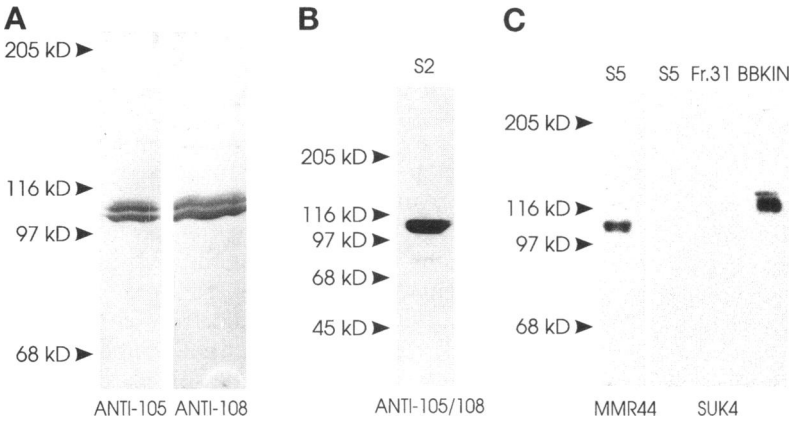

Figure 4. Immunological characterization of the *Neurospora* motor. (A) Immunoblots of S5 probed with purified anti-105-kDa and anti-108-kDa antibodies. The purified antibodies recognize both polypeptides. (B) Immunoblot of whole cell extract probed with purified anti-105-kDa antibody. No other polypeptides are recognized. (C) Immunoblots of S5, fraction 31 (Fr.31) of the gel filtration column (peak fraction of motor), and bovine brain kinesin (BBKIN) probed with peptide antibody MMR-44 and monoclonal antibody SUK4. The *Neurospora* motor is recognized by the peptide antibody (prepared against a peptide within the putative microtubule binding region of the kinesin head), but not SUK4.

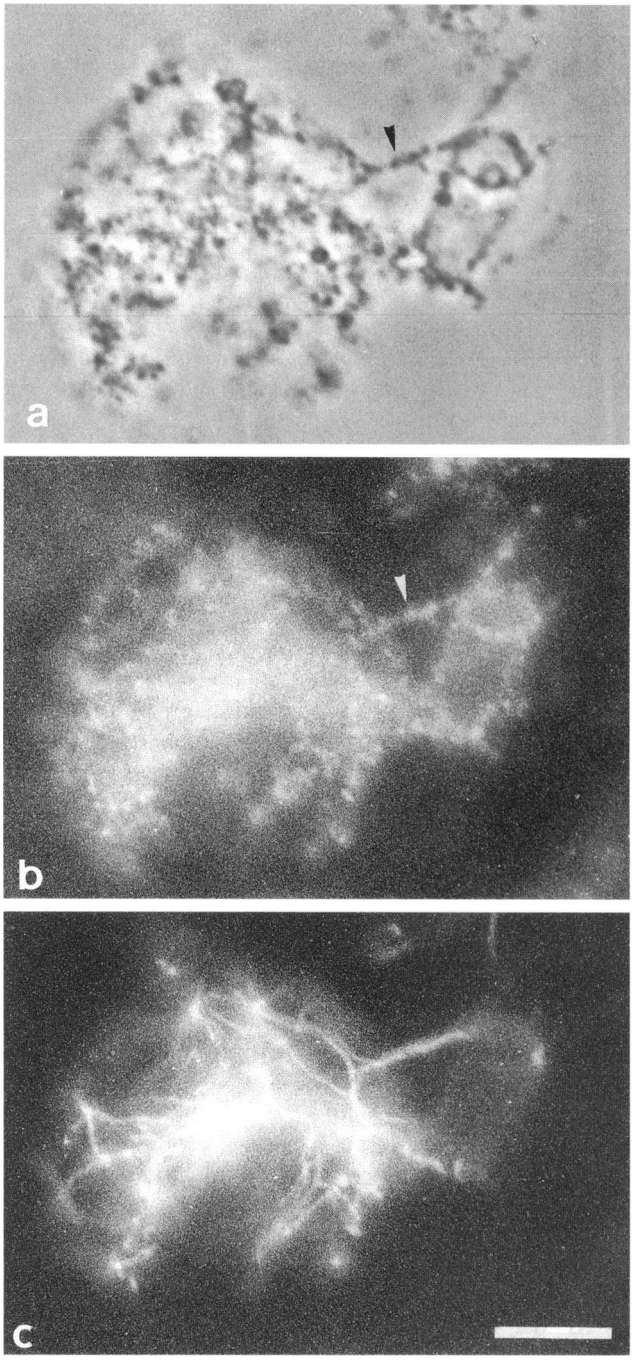

Figure 5. Immunolocalization of the *Neurospora* motor molecule in a large SLIME cell fragment adhering to a coverslip. (a) Phase contrast, (b) labeling with anti-105/108 kDa antibody, and (c) tubulin antibody. Vesicular structures, many still associated with microtubules, are labeled. Bar, 7 μm.

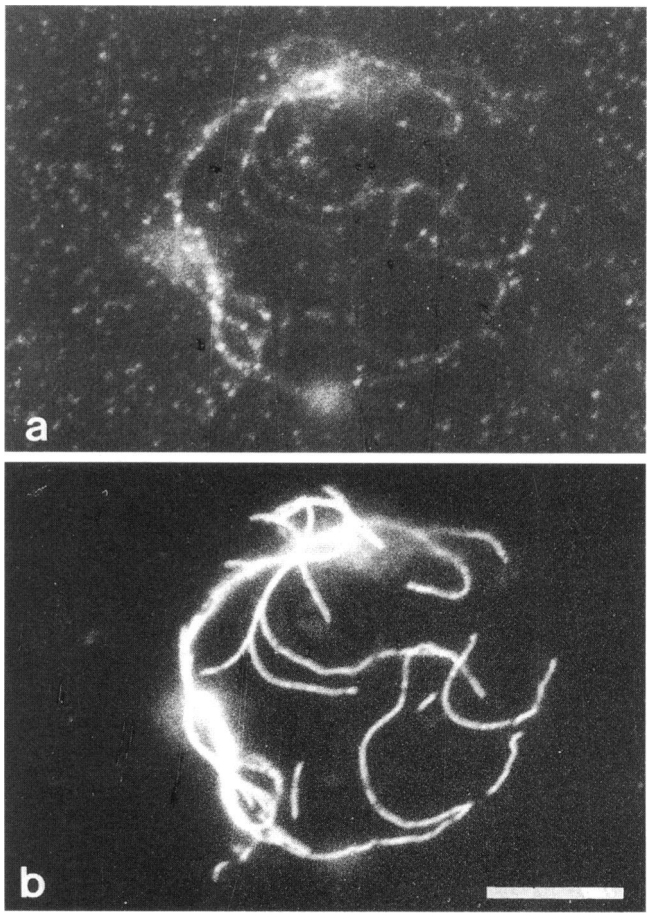

Figure 6. Higher magnification of a small, flat cell fragment stained with purified anti-105-/108-kDa antibody (a) and a monoclonal antibody against β tubulin (b). The anti-105-/108-kDa antibody labels small vesicular structures still associated with microtubules. Bar, 7 μm.

the general consensus sequence for P-loop motifs GXXXXGKT/S (Saraste *et al.*, 1990) (shaded boxes in Figure 7). 2) Like other kinesins, the middle section (stalk) of Nkin has regions predicted to form α-helical coiled-coils. However, whereas in other conventional kinesins such a region spans several hundred residues with only small discontinuities (Yang *et al.*, 1989; Navone *et al.*, 1992), the Nkin stalk has several relatively short regions predicted to form coiled-coils that are separated by non-α-helical segments (Figure 9). 3) The nonmotor domain of Nkin shows virtually no homology to other known kinesins, with the exception of a region near the C-terminus of 60–80 amino acids starting at amino acid 781 (Figure 10). This region contains a conserved 24 aa long motif near the C-terminus (aa 781–804), located in the last predicted coiled-coil segment of the molecule, that shares 50% identity and 95% similarity with all members of the family of conventional kinesins. The motif is not found in any kinesin-like protein. 4) A more detailed sequence analysis of the motor domain reveals Nkin to occupy a unique position. The amino acid identity of Nkin to the kinesin-like proteins is in the range of

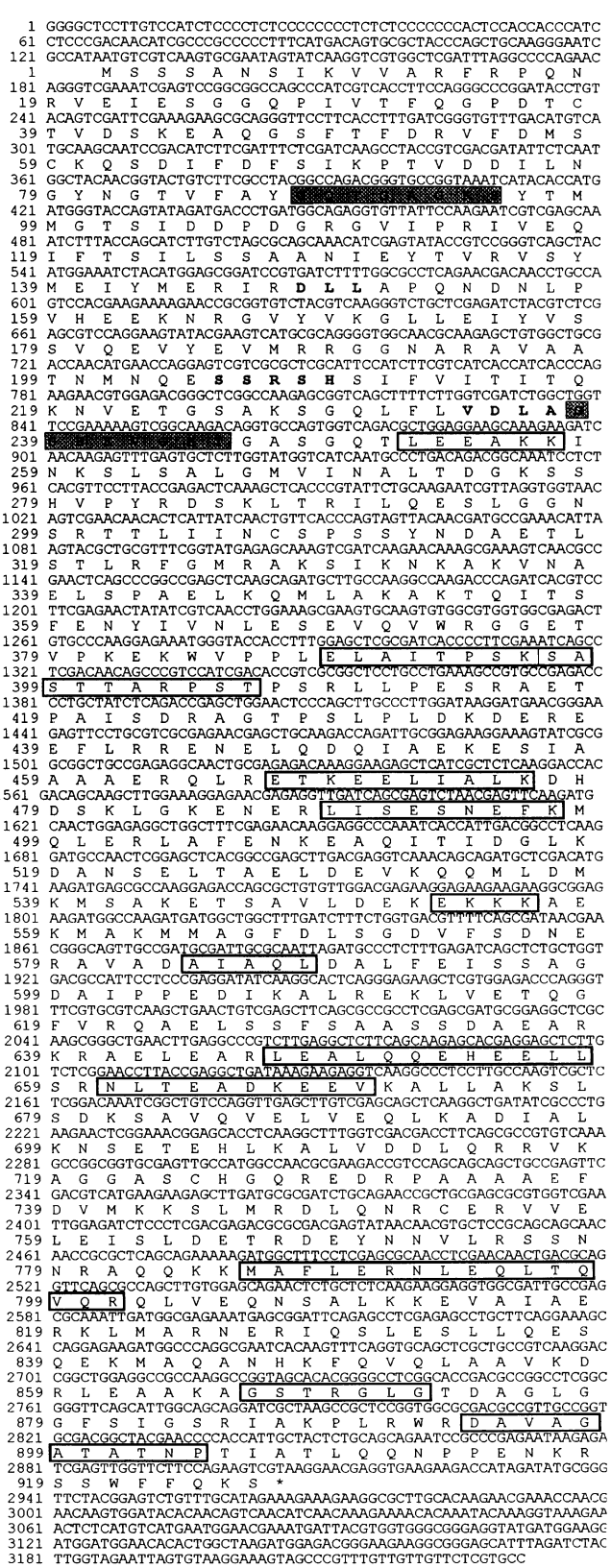

Figure 7.

30–45%, which is the same level of identity these motors display when compared with each other or with the conventional kinesins. Relative to each of the conventional kinesins, however, the motor domain of Nkin shows 53–57% amino acid identity, which is less than the conventional kinesins share with each other (> 70%), but clearly higher than that of the kinesin-like proteins.

Although this may suggest Nkin to be of a novel kinesin with "intermediate" characteristics, an analysis of the molecular phylogeny of the Nkin motor domain using the programs DARWIN (Gonnet, 1994) or PHYLIP (Felsenstein, 1989) places the *Neurospora* motor close to the base of the branch leading to the conventional kinesins (Figure 11A). Nkin therefore appears to be a distant relative of the family of conventional kinesins. We also compared the entire sequence of Nkin with the sequences of conventional kinesins using the program DARWIN. Extending off the hypothetical origin (which now represents the origin of the conventional kinesins) are two branches, with Nkin representing one branch and all other conventional kinesins representing the other branch (Figure 11B). The evolutionary distance (expressed in PAM units) from the hypothetical origin is similar for Nkin and each of the members located on the other branch.

DISCUSSION

We have cloned and partially characterized a microtubule-based motor protein from the Ascomycete *N. crassa*. The combined molecular, biochemical, and immunological evidence presented here establishes this motor molecule Nkin as a novel member of the kinesin superfamily. Molecular phylogenetic analyses of the motor domain using the programs DARWIN or PHYLIP suggest Nkin to originate near the root of the family tree of conventional kinesins, making it an intriguing addition to this otherwise homogenous motor protein family. Many of the properties of Nkin are similar to kinesins isolated from other organisms. Its size, 103 kDa, is similar to, although somewhat

Figure 7 (continued). Nucleotide and deduced amino acid sequence of NKIN. The first shaded box (residues 86–95) indicates the consensus P-loop domain present in all other kinesins. A second potential P-loop domain (also shaded) is located at residues 234–240 next to a highly conserved peptide domain (VDLAGSE). This as well as two other conserved peptides (SSRSH and DLL) are shown in bold. Peptide fragments obtained by direct peptide sequencing of isolated *Neurospora* motor protein are boxed; they are scattered throughout the sequence. The nucleotide sequence around the first methionine codon is a good match to the consensus for translation initiation in eukaryotes, including *Neurospora*. The termination codon is followed by several in-frame stop codons. As in most other *Neurospora* cDNA sequences identified so far, the poly-A tail is missing. These sequence data are available from EMBL under accession number L47106.

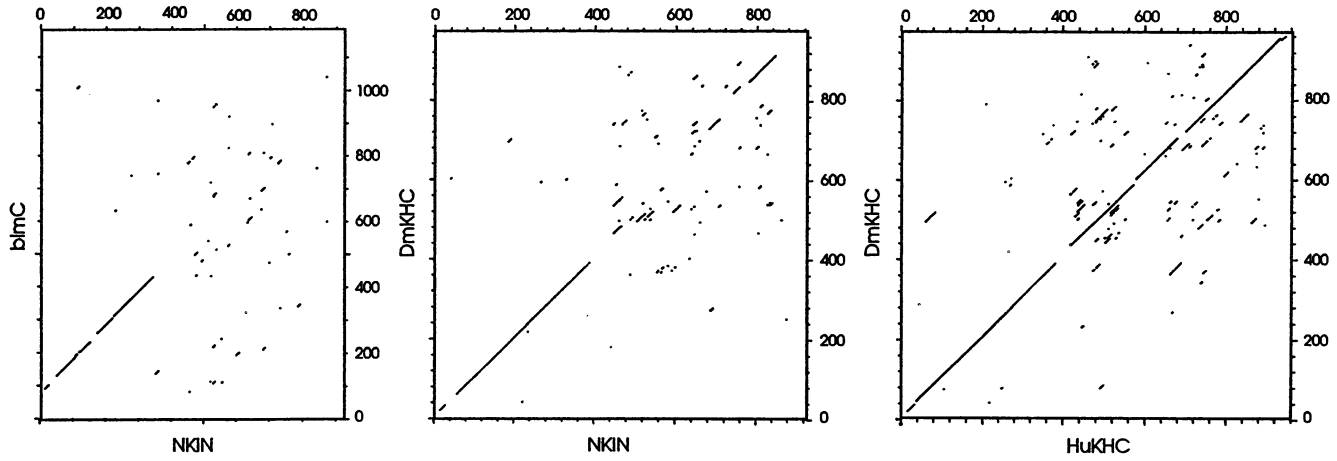

Figure 8. Dot matrix sequence comparisons of Nkin and bimC (left), Nkin and *Drosophila* heavy chain (middle), and human and *Drosophila* heavy chains (right). Although the latter two show extensive homology throughout their length, only the N-terminal motor domains exhibit striking conservation when Nkin is compared with either bimC or the *Drosophila* heavy chain. Similar plots are obtained with other kinesin-like proteins in place of bimC. Thus in overall sequence organization Nkin resembles a kinesin-like protein more than a conventional kinesin. Comparisons were done using the UWGCG program COMPARE and plotted using DOTPLOT.

smaller than, that of the heavy chains of other biochemically isolated kinesins. The motile activity consistently cofractionates with the 105-/108-kDa doublet. The other prominent polypeptide species at 180 kDa does not show motile activity in gliding assays, although the protein appears to bind microtubules in an ATP-dependent fashion. As expected, the purified motor possesses a microtubule-stimulated ATPase activity, and the polarity of movement is directed toward the plus end of microtubules. These are characteristics shared by other kinesin motors. The physiological and immunological properties of Nkin also differ from those of its higher eukaryotic counterparts in several respects. First, the velocity of microtubule movement on glass coverslips is four- to eightfold faster than the movement generated by most other kinesins in a similar assay system. Comparatively fast gliding velocities were, however, reported for microtubule motors

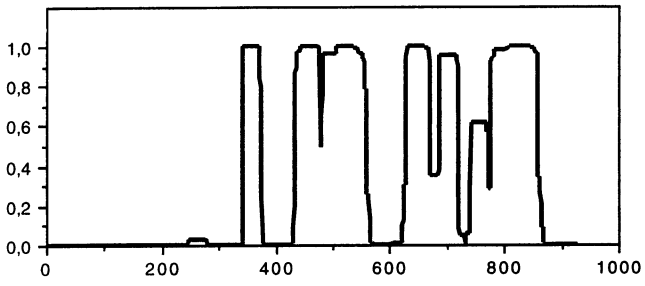

Figure 9. Predicted coiled-coil regions of Nkin using the program COILS (Lupas *et al.*, 1991) with a window size of 28 amino acids. Regions predicted to form coiled-coils are separated by longer stretches of presumably globular shape.

believed to be kinesins of the amoeba *Acanthamoeba castellanii* (Kachar *et al.*, 1987) and the slime mold *Dictyostelium discoideum* (McCaffrey and Vale, 1989). Their possible relationship to Nkin remains to be determined, but polyclonal antibodies against Nkin do not cross-react with partially purified *Dictyostelium* motor fractions (Steinberg and Schliwa, unpublished data). Second, there is no evidence for the presence of light chains or other co-purifying polypeptides. A polypeptide at about 120 kDa appears to co-migrate with Nkin after column chromatography but is clearly separated from it in sucrose density gradients. Fractions of conventional bovine brain or sea urchin kinesin without light chains have been described (Hackney *et al.*, 1991; Skoufias *et al.*, 1994), and kinesin-like proteins such as the homotetramer of fruit flies (Cole *et al.*, 1994) or the heterotrimeric kinesin of sea urchins (Cole *et al.*, 1993) likewise appear to lack light chain-like copurifying polypeptides. However, Nkin is the first motor related to conventional kinesins that is consistently isolated free of light chains. Because of the sensitivity of light chains of other kinesins to proteolysis (Kuznetsov *et al.*, 1989; Beushausen *et al.*, 1993), we cannot formally exclude the possibility that native Nkin in vivo has associated light chains. However, using isolation protocols similar to ours, others have regularly isolated a tetrameric species as native kinesin (e.g. Vale *et al.*, 1985; Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988). Unless Nkin light chains are exquisitely sensitive to proteolysis, we therefore would expect to find them using the isolation protocol employed here. Further studies on Nkin as well as other fungal sources may be necessary to clarify this point. Third,

immunological criteria clearly distinguish Nkin from other kinesins. Polyclonal antibodies raised against the 105-kDa polypeptide do not cross-react with sea urchin or bovine kinesin. Conversely, a monoclonal antibody raised against sea urchin kinesin heavy chain SUK 4 (Ingold *et al.*, 1988), which recognized other kinesin heavy chains, does not cross-react with Nkin. On the other hand, an antibody raised against a peptide from the motor domain of kinesin (Marks *et al.*, 1994) does recognize Nkin as this peptide is substantially conserved between Nkin and other conventional kinesins. By all accounts Nkin is a good candidate for an organelle motor of *Neurospora*. Affinity-purified polyclonal antibodies against the 105-kDa polypeptide stain vesicular structures in *Neurospora*. The use of small cell fragments of the wall-less SLIME mutant has allowed for better antibody localization and clearly demonstrates co-localization of many of the vesicular structures with single microtubules. Such a localization is consistent with the observation of organelle movements along microtubules in vivo (Steinberg and Schliwa, 1993). It is also consistent with localization studies using both monoclonal and polyclonal antibodies to kinesin heavy and light chains in a variety of other cell types. In most cases, these antibodies stained membraneous structures that apparently interact with microtubules (e.g., Pfister *et al.*, 1989; Hirokawa *et al.*, 1991; Wright *et al.*, 1991; Navone *et al.*, 1992). In addition, Nkin is associated with isolated membraneous organelles (microsomes and mitochondria) of *Neurospora* (Steinberg and Schliwa, unpublished data). Taken together, on the basis of these criteria Nkin is the functional homologue of the kinesin heavy chain of higher eukaryotes.

The general molecular design of Nkin is similar to that of other members of the kinesin superfamily and consists of a N-terminal motor domain, a central stalk-like domain, and a small non-α-helical (presumably globular) tail domain. Nkin appears to belong to the family of conventional kinesins, but within this family it clearly occupies the position of an outsider. This view is supported by the existence of several unique features of the Nkin sequence. The level of sequence identity to the members of this family is between 53 and 57% and thus is higher than that of all other kinesin-like proteins (30–45%), but lower than that of the conventional kinesins when compared with each other (70–80%). The motor domain thus seems to represent an intermediate between the two large classes of kinesins identified by sequence analyses (Goldstein, 1993; Bloom and Endow, 1994). Within the central stalk domain, Nkin's sequence is unique; this distinguishes Nkin from all other members of the family of conventional kinesins, which show about 45% sequence identity even within the stalk domain. Although unique, the stalk domain of Nkin has regions that have a high probability of forming a coiled-coil; however, these regions are shorter and more widely separated from each other than in other members of this family (Yang *et al.*, 1988). This may result in a less elongated, more compact shape of the native molecule.

Nkin and the group of conventional kinesins of animal species are located on the same branch of the phylogenetic tree (Figure 11), suggesting a common ancestry. A phylogenetic relationship of Fungi and Animalia is, in fact, consistent with current phylogenetic frameworks inferred from comparisons of protein and rRNA sequences (Baldauf and Palmer, 1993; Wainwright *et al.*, 1993). According to maximum likelihood analysis, animals and fungi share a common evolutionary history, having diverged from a uniflagellated protist about 1200 million years ago (Wainwright *et al.*, 1993, 1994). In light of this phylogenetic relationship, the lower percentage of homology within the motor domain between Nkin and other conventional kinesins (about 55%) is consistent with the evolutionary process. The "common ancestor" of *Drosophila* and humans, whose kinesin motor domains are about 75% identical, lived about 600 million years ago (Stanley, 1986). Assuming a constant molecular clock for the motor domain, an identity of ~55% is what one would expect for two motors (species) that diverged ~1200 million years ago.

The two most conspicuous features of the Nkin sequence are the presence of a second P-loop motif in the motor domain and a short stretch of high sequence homology between Nkin and all other members of the conventional kinesins near the tail domain. P-loop motifs in molecular motors attract attention because they may have a role in the generation of force or other aspects of motor function such as binding to microtu-

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NKIN     GGASCHGQRED----------RPAAAAEFDVMKKSLMRDLQNRCERVVELEIS-----LD 764
DmKHC    MTADYEKVRQEDAEK-SSELQNIILTNER--------REQARKDLKGLEDTVAKELQTLH 818
Unc116   AKESSTAAEHDAEAKLAADVARVESGQDAGRMKQLLVKDQAAKEIKPLTDRVNMELTTLK 666
                 ..                .         ..   .  .  .     *

NKIN     ETRDEYNNVLRSS--------------NHAQQKKMAFLERNLEQLTQVQRQLVEQNSAL 810
DmKCH    NLRKLFVQDLQQRIRKNVVNEESEEDGGSLAQKQKISFLENNLDQLTKVHKQLVRDNADL 878
Unc116   NLKKEFMRVLVARCQANQ-DTEGEDSLSGHAQKQRIQFLENNLDKLTKVHKQLVRDNADL 725
         . .  .  *                    ********** ********** .*. *

NKIN     KKEVAIAERKLMARNERIQSLESLLQESQEKMAQANHKFQVQLAAVKDRLEAAKAGSTRG 870
DmKHC    RCELPKLEKRLRCTMERVKALETALKEAKEGAMRDRKRYQYEVDRIKE------AVRQKH 932
Unc116   RVELPKMEARLRGREDRIKILETALRDSKQRSQAERKKYQQEVERIKE------AVRQRN 779
         . *..  * .*    .*.. **. *.....       ..* ..  .*.      *   .
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Figure 10. Sequence comparison of the C-terminus of Nkin with that of human (HuKHC) and *Drosophila* (DmKHC) kinesin, showing a region of high sequence identity (boxed) embedded in a larger segment of moderate to low sequence identity. The underlined amino acid sequence was also identified by peptide sequencing of biochemically purified motor.

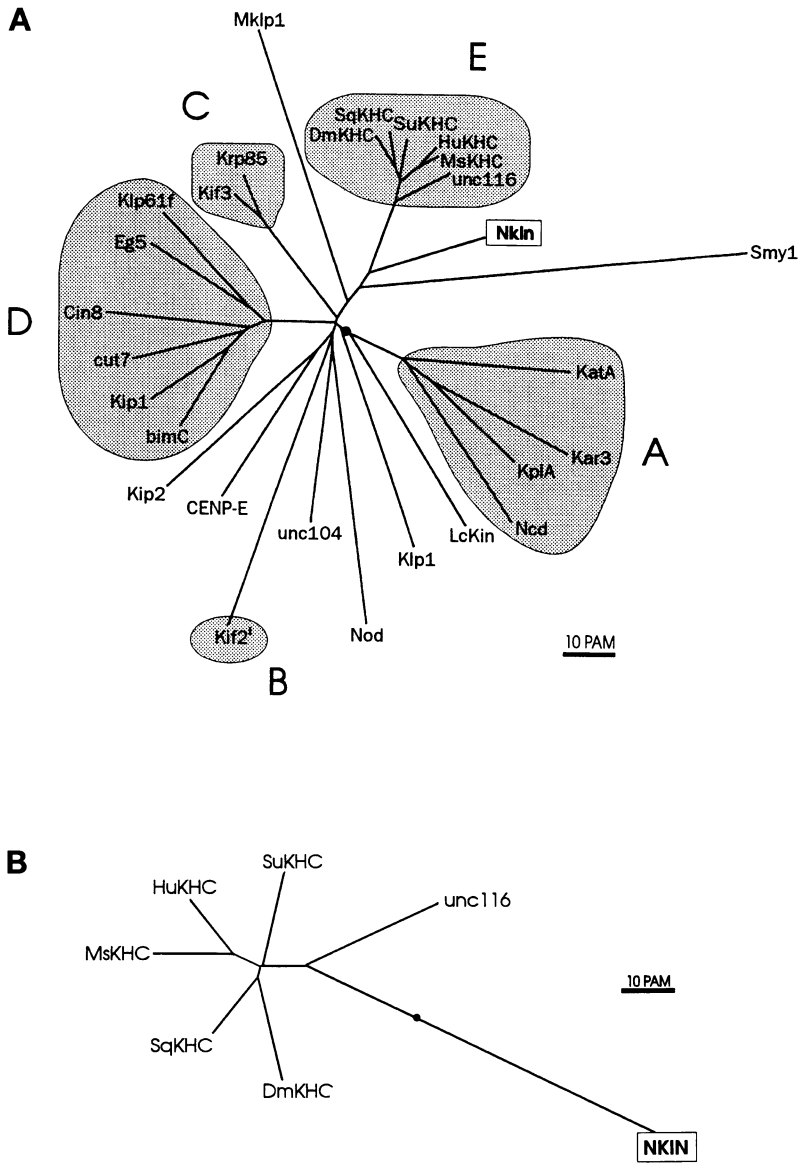

Figure 11. (A) Phylogenetic tree of 28 representative kinesin motor domain amino acid sequences. Kinesin families are shaded and designated A through E according to Goodson *et al.* (1994). The tree is drawn unrooted; the hypothetical origin is marked with a small black dot. According to this analysis, Nkin is a member of the conventional kinesins, but it branches off the family tree close to the base (prepared with the program DARWIN). Evolutionary distance is given in PAM (point accepted mutation) units. (B) Phylogenetic tree of the entire amino acid sequences of the conventional kinesins, including Nkin. From the hypothetical origin two branches arise, one representing Nkin and the other branch representing the rest of the members of this family. This was prepared with the program DARWIN. Evolutionary distance is given in PAM units.

bules or regulation of activity. Both axonemal and cytoplasmic dyneins are known to have four P-loop motifs, one of which is believed to have a primary function in ATP hydrolysis and force generation (Gibbons *et al.*, 1991; Koonce *et al.*, 1992; Vallee, 1993). Whether the other three represent functional binding sites for ATP or other nucleoside triphosphates is unknown, as is their possible function. The same is true for the second P-loop motif in Nkin, which fits the general consensus sequence of P-loop motifs but lacks the glycine in position 4 found in P-loop motifs of motor proteins. Conceivably, the second motif of Nkin could have arisen by a random mutation and have no function. Fortuitous P-loop motifs in proteins with no known NTP-binding activity are known (E. V. Koonin, personal communication). However, the second P-loop of Nkin presumably is exposed on the surface and occurs in a domain whose sequence is otherwise highly conserved among the conventional kinesins (100% identity within a 20-amino acid stretch surrounding this motif). Clearly, further work is necessary to determine whether secondary P-loops might occur in other organelle motors of lower eukaryotes and what their function might be.

The second conspicuous sequence motif to emerge from the sequence analysis is a stretch of 24 amino acids near the C-terminus that exhibits 65% identity and 95% similarity to the corresponding regions in all

other conventional kinesins. It is part of a larger region of more moderate sequence homology. What makes this region so intriguing is the fact that it is the only stretch of homology between Nkin and the conventional kinesins outside the motor domain. Because a similar region of homology is not found in kinesin-like proteins, this domain seems to serve a biologically important function in Nkin as well as the conventional kinesins and supports the notion that Nkin is a member of this family. Possible functions include the following: 1) A role in dimerization. The conserved motif is present in a segment of the molecule predicted to form a coiled-coil. Because the coiled-coil stalk is generally believed to be involved in dimerization, this segment, among others, could contribute to the formation of a homodimer. The question that remains to be answered, then, is why this and only this segment of the coiled-coil stalk region has been so highly conserved between Nkin and the other members of this family. 2) A role in the regulation of motor activity. In conventional kinesins, the stalk domain is interrupted near its middle by a proline-containing stretch that may contribute to a "folded" configuration of the native molecule where the tail may be brought in contact with the motor domain (Hackney *et al.*, 1992). It is conceivable, although not demonstrated, that this may influence motor activity, and may be mediated by the conserved tail domain. 3) Binding to light chains. Kinesin light chains bind near the C-terminal region of the heavy chain. Binding is tight and may be mediated by coiled-coil domains (Cyr *et al.*, 1991). A stretch of heptad repeats N-terminal to the globular tail domain of *Drosophila* kinesin, which excludes the conserved motif identified here, has been implicated in heavy chain-light chain interaction (Gauger and Goldstein, 1993). Because the precise site of association is not known, a contribution of the conserved motif cannot be ruled out with certainty. 4) Binding to organelles (cargos). Even in the absence of light chains, the tail of kinesin heavy chain has intrinsic membrane binding ability (Skoufias *et al.*, 1994). Because Nkin and conventional kinesins have in common that they are motors for certain types of membraneous organelles, it is conceivable that the conserved tail region (including the 24-aa motif) contributes to membrane binding. Further studies are under way to distinguish between these possibilities.

In conclusion, we have identified a new member of the kinesin superfamily that exhibits several unique features. It appears to be a distant relative of the conventional kinesins, an otherwise extremely coherent, highly conserved protein family. Nkin may change the way we view this motor family and may offer novel opportunities for the study of kinesin organelle motors.

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