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Functional anatomy of the kinesin molecule *in vivo*

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We have developed an assay that allows the functional efficiency of mutant kinesins to be probed *in vivo*. We show here that the growth rate of the filamentous fungus *Neurospora crassa* can be used as a sensitive reporter for the ability of mutant kinesins to suppress the phenotype of the kinesin null mutant of *Neurospora*. Truncation mutants, internal deletion mutants and chimeras, in which homologous domains were exchanged between different fungal kinesins, were generated and transformed into the kinesin-deficient strain. None of the mutations affect motor velocity *in vitro*, but even minor alterations in the tail domain severely compromise kinesin's performance *in vivo*. The analysis of these mutants has identified subdomains in the stalk and tail likely to be involved in cargo binding and/or regulation of motor activity. The phenotypes of several mutants strongly suggest that kinesin requires a folded conformation to achieve full functionality *in vivo*. Folding critically depends on two flexible domains in the stalk that allow an interaction of the tail with the neck/hinge region near the catalytic motor domain. The assay has proven to be a valuable tool in the analysis of kinesin function *in vivo* and should help to characterize the sites involved in intra- and intermolecular interactions.

Keywords: chimeras/folding/kinesin/mutagenesis/ *Neurospora crassa*

Introduction

Motor proteins of the kinesin superfamily use the energy derived from ATP hydrolysis to move along microtubules (for reviews, see Bloom and Endow, 1994; Vale and Fletterick, 1997). The best-studied class of kinesin motors is conventional kinesin. It was purified originally from squid and bovine brain (Brady, 1985; Vale *et al.*, 1985) and sea urchin eggs (Scholey *et al.*, 1985), and is now known to be present in many organisms ranging from humans to fungi (for a review, see Hirokawa, 1998). Conventional kinesins isolated from animal species are heterotetramers consisting of two heavy chains (100–130 kDa) and two light chains (60–70 kDa). None of the fungal kinesins has been reported to have light chains. Based on rotary shadow electron microscopy images, conventional kinesin is an elongated molecule with two globular heads at the N-terminus, followed by a stalk that is sometimes kinked in the middle, and a fan-shaped tail (Hirokawa *et al.*, 1989; Hackney *et al.*, 1992; Bloom and Endow, 1994).

The globular head is the best characterized domain of the kinesin molecule (for reviews, see Howard, 1996; Vale and Fletterick, 1997). It possesses the nucleotide-binding site and the microtubule-binding interface (e.g. Yang *et al.*, 1989; Gilbert *et al.*, 1995; Woehlke *et al.*, 1997) and generates the conformational change that initiates a 'step' on the microtubule lattice. Recent studies demonstrate that the two domains adjacent to the catalytic motor domain, termed neck and hinge, are also essential for efficient motor function. They are involved in the determination of the directionality of movement along the microtubule (Case *et al.*, 1997; Henningsen and Schliwa, 1997; Endow and Waligora, 1998), processivity (Romberg *et al.*, 1997) and mechanochemical coupling (Grummt *et al.*, 1998b). Thus the functional motor domain includes not only the globular head but also the adjacent neck and hinge regions. Much less is known about the other subdomains of the kinesin molecule that make up the stalk and tail. Following the hinge, they include a coiled-coil domain termed coil 1, a flexible kink and a second coiled-coil domain (coil 2; see also Goldstein, 1993). Another, presumably flexible, linker region separates coil 2 from the tail, which consists of a coiled-coil domain and the globular C-terminus (see Figure 3).

We have set out to obtain a detailed functional map of the non-motor portions of the kinesin molecule. To this end, we have taken advantage of the filamentous fungus *Neurospora crassa* as a model system. In contrast to kinesin heavy chain knockout mutants in the mouse (Tanaka *et al.*, 1998) and *Drosophila* (Gho *et al.*, 1992), which were lethal, the deletion of conventional kinesin in *N.crassa* leads to a viable phenotype characterized by contorted growth, a defect in the transport of secretory vesicles to the tip and a dramatically reduced linear growth rate (Seiler *et al.*, 1997). The *Neurospora* null mutant allowed us to develop an effective assay for kinesin function *in vivo*. We show here that the growth rate can be used as a sensitive reporter of the functional fidelity of mutant kinesins generated by rational design and transfected into the null mutant. Using this assay, we have determined the rescue potential of truncation mutants, internal deletion mutants and chimeras where subdomains of *Neurospora* kinesin were substituted with the corresponding domains of other kinesins. Thus, we were able to obtain information on the contributions of kinesin domains in the stalk and tail, whose function is best studied in an *in vivo* context. The analysis of these mutants has identified regions of the molecule likely to be involved in cargo binding and regulation, and further suggests that kinesin adopts a folded conformation *in vivo*.

Construct	Growth Rate in cm/day	Rescue of NcKin⁻ in %
NcKin WT	6.8 ±0.5	—
NcKin rescue	7.1 ±0.3	100
NhKin	5.4 ±0.1	75
SrKin	2.4 ±0.4	20
UmKin	2.0 ±0.4	10
DmKhc	no protein expressed	
NcKin⁻	1.4 ± 0.3	0

Fig. 1. Rescue of the NcKin null mutant by all four fungal conventional kinesins. The constructs were cloned behind an *Aspergillus* TrpC promotor and expressed in the NcKin null mutant together with a bleomycin resistance cassette. The NhKin construct was a fragment of *Nectria* genomic DNA in a λ Zap vector. Positive clones were placed into a race tube, and longitudinal growth was measured. The growth rate of the fastest clones is depicted in the figure.

Results

Suppression of the growth defect of the Neurospora crassa kinesin null mutant

We first expressed all four known fungal conventional kinesins in the *Neurospora* kinesin null mutant. Fungal conventional kinesins form a distinct subgroup of the family of conventional kinesins (Hirokawa, 1998) that share certain sequence motifs not found in other conventional kinesins. There are four fungal conventional kinesins known so far, the best characterized being that of the ascomycete *N.crassa* (NcKin; Steinberg and Schliwa, 1995, 1996). The others were isolated from its close relative *Nectria haematococca* (NhKin; Wu *et al.*, 1998), also an ascomycete, the plant-pathogenic basidiomycete *Ustilago maydis* (kin2, or UmKin; Lehmler *et al.*, 1997; Steinberg *et al.*, 1998) and the zygomycete *Syncephalastrum racemosum* (SrKin; Steinberg, 1997; Grummt *et al.*, 1998a). The results are shown in Figure 1. NcKin rescues the null mutant completely (7.1 cm/day), while transformation of NhKin, SrKin and UmKin results in a partial rescue. We also attempted to express *Drosophila* kinesin in the null mutant. Though resistant clones were obtained and the gene was present (tested with PCR on genomic DNA using DmKHC-specific primers), no protein was expressed. The rescue of the deletion mutant is not an all-or-none phenomenon but a graded response that apparently depends on the degree of sequence similarity to NcKin.

We then constructed a set of modified kinesins in the null mutant of *N.crassa*, and have used the growth rate measured in race tubes as an assay for the functional rescue of the null phenotype. These mutants are analysed in detail below. The mutants investigated in this manner displayed a spectrum of growth rates, indicating this selection scheme to be a sensitive way of screening for kinesin's *in vivo* functionality. To our knowledge, the *Neurospora* system is unique in this respect and currently is the only cell model in which kinesin function can be studied using a quantitative *in vivo* assay.

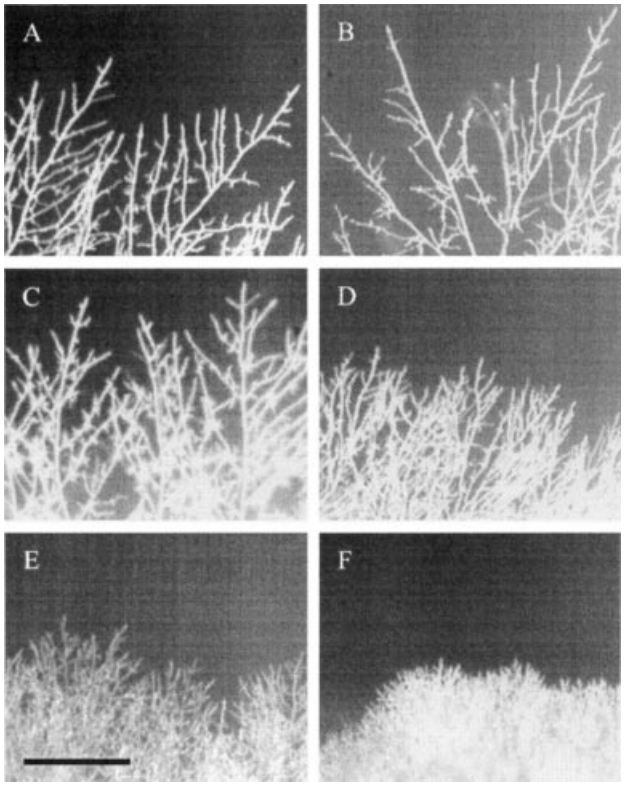

Fig. 2. Colony morphologies of clones transformed with different kinesin constructs. (**A**) Wild-type (growth speed: 7 cm/day), (**B**) clone showing full rescue (7 cm/day), (**C**) clone showing good rescue (5.5 cm/day), (**D**) clone showing intermediate rescue (4 cm/day), (**E**) clone showing poor rescue (2.5 cm/day) and (**F**) clone showing no rescue (1.5 cm/day). This clone has the same morphology as the NcKin null mutant. Bar: 1 mm.

The growth rate assay requires a careful clonal selection, because not every transformed, bleomycin-resistant clone expresses the desired protein, and because growth rates may range between that of the null mutant and the optimal growth rate for a given construct. We therefore screened at least 30 independent clones for each construct (for details, see Materials and methods). In transformations with NcKin wild-type, we regularly obtained transformants growing at wild-type speed, testifying to the reliability of the selection process.

As outlined in Materials and methods, the expression levels of mutant kinesins are not rate-limiting. In addition, all mutant proteins (except NcΔ740, Figure 5-8 which did not adhere to glass but still bound to microtubules) showed a gliding velocity expected for fungal kinesins, >2 μm/s. Thus all constructs possessed mechanochemical activity equivalent to that of wild-type kinesin. We also studied the morphology of hyphae at the leading front of growing colonies and the appearance of the *Spitzenkörper*, an accumulation of vesicles in the hyphal apex linked to secretion and cell growth. Colony morphology was found to correlate with the growth rate of a given construct but seemed to be independent of the exact nature of the mutation introduced. A range of typical colony morphologies is presented in Figure 2. A *Spitzenkörper* could only be found in clones with full or nearly full rescue capacity, but not in clones growing more slowly than 4 cm/day (not shown).

In conclusion, rescue of the growth rate appears to be

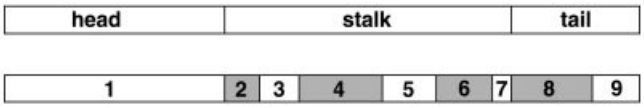

Fig. 3. Overview of the domains of fungal conventional kinesins. (1) Catalytic motor domain (amino acids 1–340 in NcKin), (2) neck (340–370), (3) hinge (370–430), (4) coil 1 (430–550), (5) kink (presumably flexible, 550–630), (6) coil 2 (630–720), (7) stalk–tail linker (presumably flexible, 720–740), (8) coiled-coil tail (740–860) and (9) globular tail (860–928). The first three domains together constitute the mechanochemically functional motor domain (Grummt *et al*., 1998b). The reason that domains 8 and 9 are designated as tail domains is based in part on previous studies (Steinberg and Schliwa, 1995) and on evidence presented here. This domain organization applies to all fungal kinesins and can also be extended, with only minor modifications, to their animal counterparts.

a simple, convenient and reliable assay for the functionality of mutant kinesins. Irrespective of the type of cargo transported or the nature of interactions with other proteins, the assay provides detailed information on the molecular structure requirements for *in vivo* function.

C-terminal deletion mutants

Based on the work of many groups (for reviews, see Vale and Fletterick, 1997; Mandelkow and Johnson, 1998), the kinesin heavy chain can be subdivided into structurally distinct domains according to the alternate occurrence of coiled-coil and flexible or globular domains. The organization of these domains, drawn to scale for NcKin, is shown in Figure 3 for future reference. Because of the suspected importance of the kinesin tail (domains 8 and 9) for cargo binding, we have first concentrated on a mutational analysis of these domains. Figure 4 shows a sequence comparison of the C-termini of three representative animal and fungal conventional kinesins which reveals some interesting similarities and differences between the two groups. First, the position of the stalk–tail linker (domain 7) that follows coil 2 is not conserved between fungal and animal kinesins. It is shifted towards the N-terminus in fungi and closer to the C-terminus in animals. Secondly, the region that runs from the beginning of the fungal to the end of the animal stalk–tail linker is highly conserved within each subfamily, but markedly different between. Thirdly, the ~70 amino acids that follow (highlighted in gray in Figure 4) are well conserved between all conventional kinesins, with relatively few class-specific residues that cluster in the C-terminal half of this region. Finally, in the globular tail domain, two highly conserved motifs stand out, one found in all kinesins (IAKPL/IR) and the other specific for the C-terminus of fungal kinesins (KRSSW). Details of the differences between the two groups of conventional kinesins will be considered later, but this overview may help to orient the reader in the discussion of the deletion constructs that follow.

Stepwise deletion from the C-terminus reveals several interesting features. Unexpectedly, a deletion of only five amino acids from the C-terminus already leads to a decrease in rescue of >50% (construct NcΔ923, Figure 5-1; for construct nomenclature, see Materials and methods) although the protein is very abundant. Further stepwise deletion of the globular tail domain and the C-terminal 30 amino acids of the coiled-coil tail does not alter the growth rate markedly (constructs NcΔ907–NcΔ830). In contrast, deletion of at least half of the tail coiled-coil in constructs NcΔ801–NcΔ740 (Figure 5) results in complete loss of functionality. This loss of the ability to rescue coincides with the beginning of the most conserved part of the tail coiled-coil (see Figure 4).

C-terminal chimeras between NcKin and other fungal kinesins

To explore whether motifs in the tail region of SrKin and NhKin are important for the decrease in growth speed observed in these constructs (see Figure 1), we used chimeras between NcKin and these kinesins to study further the function of the tail (Figure 6). In contrast to deletions, in these chimeras the overall architecture of the molecule remains unchanged, and domains that are considered to be structurally and functionally important can be exchanged intact. Furthermore, the sequence differences between swapped domains may help to pinpoint amino acid positions of particular importance.

Replacing the NcKin tail (domains 8 and 9) with the tail of the closely related ascomycete *N.haematococca* results in a chimera that fully rescues the NcKin null mutant (Nc 741/741 Nh, Figure 6-1). In contrast, a corresponding construct with the tail from the zygomycete *S.racemosum* (Nc 741/754 Sr, Figure 6-2) behaves just as full-length SrKin (Figure 1) and rescues the phenotype only marginally. These data emphasize the importance of the C-terminus for functional fidelity and suggest residues that are conserved between NcKin and NhKin, but not between NcKin and SrKin, to play an important role in the function of the tail. As can be seen in Figure 4, there are two clusters of residues conserved only between NcKin and NhKin, at positions 835–870 and 923–928, respectively, of NcKin. The corresponding insertion chimeras (Figure 6-4 to 6-6) show that the first of these stretches contains a motif important for optimal function. Even in the highly conserved domain 8 (80% sequence identity between SrKin and NcKin), the small differences that exist are sufficient to cause a decrease in rescue. The effect of swapping a small portion of the globular tail domain downstream from the conserved RIAKPL motif is even more dramatic (Nc 897/904 Sr, Figure 6-7; and Nc 920/925 Sr, Figure 6-8). Remarkably, replacing only the last five amino acids of NcKin with the corresponding piece of SrKin (construct Nc 920/925 Sr, Figure 6-8) severely reduces the activity of NcKin, corroborating the finding with the equivalent deletion construct (NcΔ923, Figure 5-1).

To determine whether the tail is the only region responsible for the decreased growth rate of SrKin, we made a series of chimeras with an SrKin backbone, including the 'reverse' constructs of Nc 741/754 Sr (Figure 6-2) and Nc 713/713 Sr (Figure 6-3). As the constructs Sr 905/900 Nc (Figure 6-9) to Sr 712/714 Nc (Figure 6-11) show, regions outside the tail domain also contribute to SrKin's lower rescue capacity. Growth speed was still markedly reduced, although introduction of an NcKin tail into SrKin resulted in an unmistakable gain of function (Sr 751/740 Nc, Figure 6-10; Sr 712/714 Nc, Figure 6-11). Thus, while there seems to be a protein–protein interaction in the tail that involves a NcKin-specific contact site, a compatible tail alone is not sufficient for full functionality.

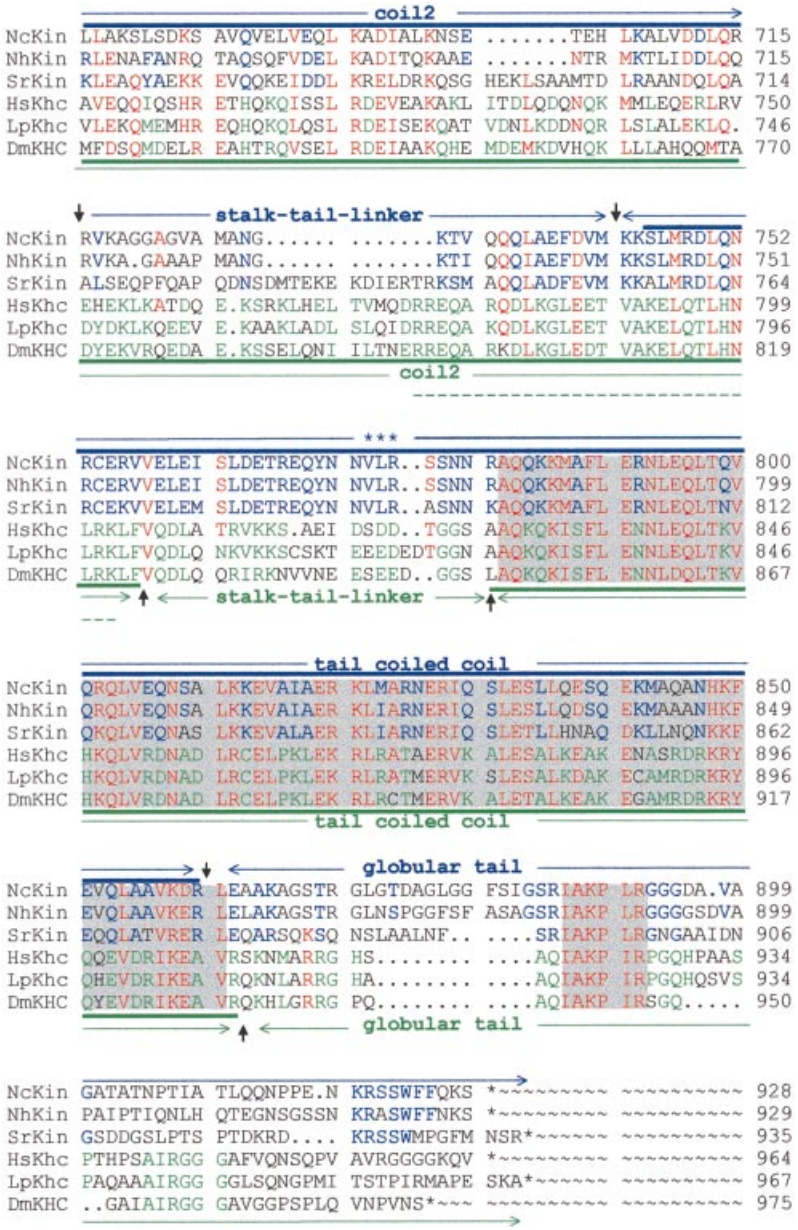

Fig. 4. Sequence alignment of three fungal (N.c., N.h., S.r.) and three animal (H.s. KIF5b, L.p., D.m.) conventional kinesins. Residues conserved among all kinesins are depicted in red, residues conserved only within the animal kinesins are depicted in green, and residues conserved only in fungal kinesins are shown in blue. Non-conserved residues are in black. Two regions of high conservation within the C-terminus are highlighted in gray. Vertical arrows show domain boundaries between coiled-coil and non-coiled-coil regions. The three blue asterisks mark a discontinuity in the heptad repeats of all fungal kinesins. The green dashed line marks the light chain-binding site identified by Diefenbach *et al.* (1998) in human kinesin. For further discussion, see text.

Head and stalk chimeras between SrKin and NcKin

The incomplete rescue of constructs Sr 751/740 Nc and Sr 712/714 Nc (Figure 6-10 and 6-11) shows that non-conserved sequence motifs important for *in vivo* function must be located in domains 1–6. To locate them, we made a series of exchanges in both the head and stalk domains (Figure 7). Again, chimeras rather than deletion mutants were used to maintain the overall architecture of the molecule. All chimeras included the NcKin tail because of the unequivocal importance of this domain for *in vivo* function.

Replacing the NcKin catalytic motor domain with that of SrKin (Sr 324/326 Nc, Figure 7-1) fully retained wild-type characteristics in the chimera. In contrast, the 'reverse' construct with a NcKin head and a SrKin neck/hinge/stalk (Figure 7-2) was indistinguishable from a construct (Figure 6-10) where all these domains were derived from SrKin. Replacing the kink, coil 2 and stalk–tail linker with SrKin domains (Figure 7-3) resulted in full rescue of the deletion mutant, while again, the 'reverse' construct did not (Figure 7-4). These four constructs draw attention to the neck and hinge regions. However, inserting the NcKin neck and hinge domains either alone (Figure 7-5 and 7-6) or in combination (Figure 7-7) into SrKin did not result in full rescue, with the combination pointing to a possible additive effect. Interestingly, though, essentially complete rescue was observed when, in addition to the neck and hinge, the stalk–tail linker was also derived from

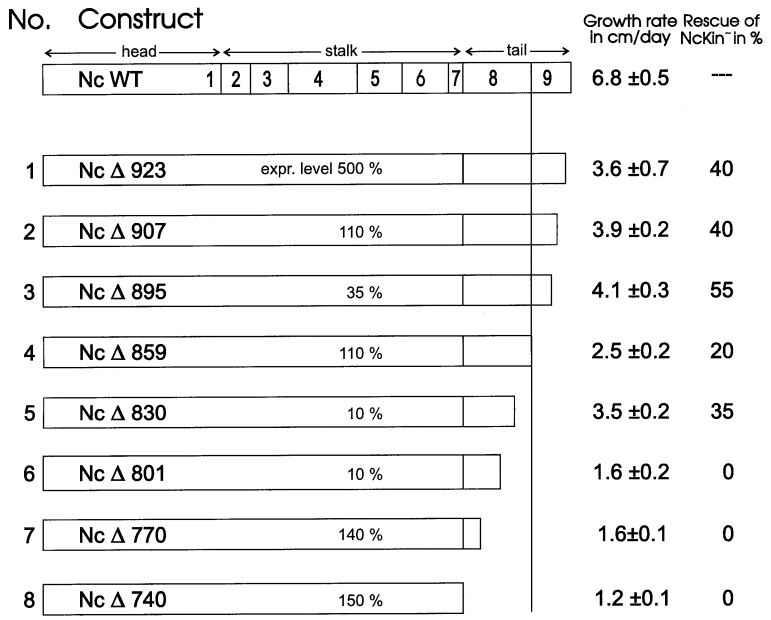

Fig. 5. Rescue by C-terminal deletion constructs. The beginning of the tail is marked in each construct and the continuous vertical line represents the boundary between the C-terminal coiled-coil and the globular domain (see Figure 3). The constructs were cloned behind the original NcKin promoter and expressed in the NcKin null mutant together with a bleomycin resistance cassette. The expression levels (see Materials and methods) of the mutant kinesins as a percentage of wild-type are also shown.

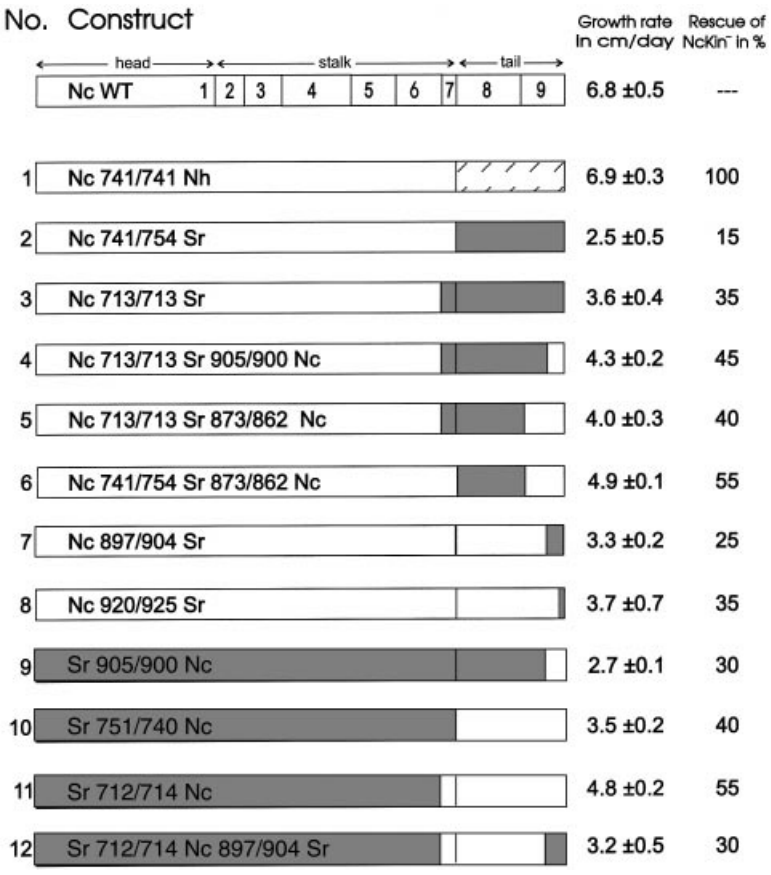

Fig. 6. Rescue by NcKin/SrKin and NcKin/NhKin C-terminal chimeras. Nomenclature: initials of first species + number of last amino acid at fusion site/number of first amino acid at fusion site + initials of second species. For discussion see text.

NcKin (Figure 7-8). This may be linked to the fact that the stalk–tail linker of SrKin is longer that that of NcKin (Figure 4). Indeed, replacing just this small domain of NcKin with the longer domain of SrKin already compromised the *in vivo* function of the motor (Figure 7-9). An even stronger effect was observed when the stalk–tail linker was deleted (NcΔ716–743, Figure 7-10). Because of the importance of the flexible stalk–tail linker (Figure 7-

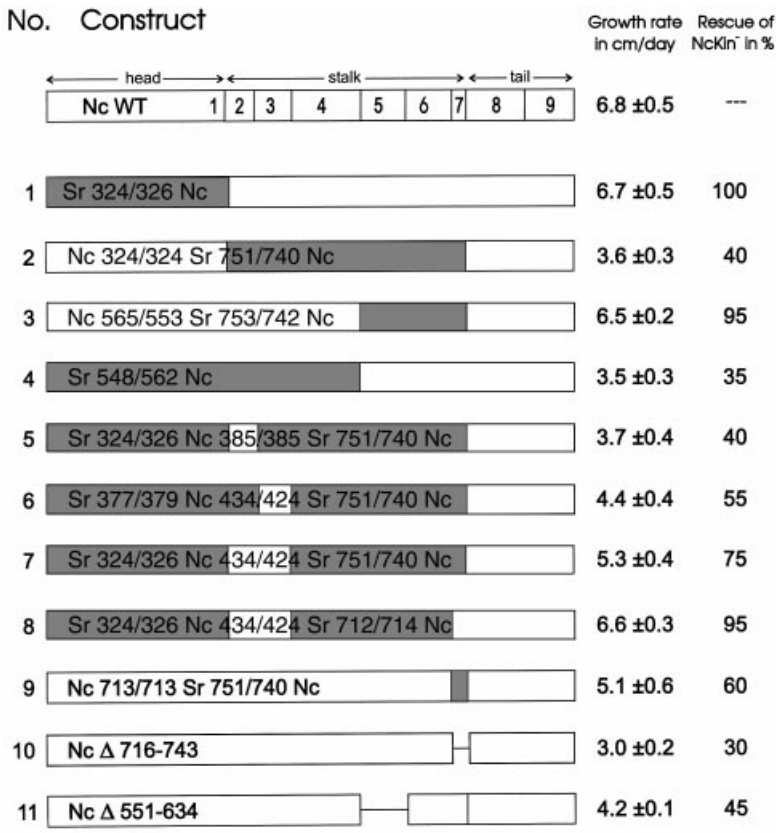

Fig. 7. Rescue by NcKin–SrKin head–stalk chimeras. For discussion see text.

9), we also probed the functional importance of the largest flexible region, the kink (domain 5). This domain was deleted, taking care to join the coil 1 and coil 2 regions in such a way that one long, continuous coiled-coil was formed. The deletion of the kink decreased the functional fidelity of the motor and led to an intermediate rescue phenotype (NcΔ551–634, Figure 7-11).

The apparent contradiction between a construct where the stalk–tail linker was replaced along with kink and coil 2 (Figure 7-3) and a replacement of the stalk–tail linker alone (Figure 7-9) seems to be due to the slightly different geometries of NcKin and SrKin. In a coiled-coil prediction, NcKin's flexible, non-coiled-coil regions differ in length from those of SrKin. It is conceivable, therefore, that these differences are tolerated and 'cancel out' in a construct where all three domains are exchanged (Figure 7-3) but not in constructs where the stalk–tail linker and the kink/coil 2 are derived from two different kinesins (Figure 7-9).

Discussion

We have established a quantitative *in vivo* assay to probe the functional importance of kinesin domains located in the stalk and tail regions. The *N.crassa* system offers this unique feature because the NcKin knockout mutant is still viable. Since the null mutant is defective in longitudinal growth (Seiler *et al.*, 1997), it is justified to use the growth rate as a key parameter of kinesin function. The analysis of a series of kinesin mutants has allowed us to gain insights into the functional importance of the domains that comprise the stalk and tail.

The tail of conventional kinesin has long been suspected to be responsible for cargo binding (Hirokawa *et al.*, 1989; Goldstein, 1993), although the regions involved could not be specified. The C-terminal deletion constructs show that all functions of cargo binding apparently reside in the tail domain and provide clues that allow a distinction between regions primarily concerned with binding and those primarily concerned with regulation. The ability to complement the null phenotype is abolished in all constructs shorter than 830 amino acids (NcΔ801 to NcΔ740), while deletions located more C-terminal to this point (NcΔ923 to NcΔ830) result in an intermediate phenotype. Thus the region preceding amino acid 830 most likely contains a key element of the cargo-binding site. Interestingly, this region is the most highly conserved motif in animal and fungal kinesins outside the catalytic motor domain. It is an attractive speculation, therefore, that the cargo-binding site and the mechanisms of cargo association are conserved between fungal and animal kinesins.

The primary role of the C-terminal amino acids downstream from residue 830 can not be in cargo binding since even complete deletion only results in an intermediate phenotype. Yet the tail is important in its entirety since the alteration or deletion of only the last five amino acids (Figures 5-1 and 6-8) already compromises its function. We suggest that the last 100 amino acids of NcKin are important for kinesin regulation. Such a role is supported by constructs Nc 741/754 Sr (Figure 6-2) and Sr 751/740 Nc (Figure 6-10), where the tail domains were exchanged between NcKin and SrKin. Both constructs are unable to rescue completely, though a gain of function is observed if SrKin is equipped with an NcKin tail (Figure 6-10).

Since both tail domains are highly homologous in the presumptive cargo-binding region, the remainder of the tail may be involved in a step (or steps) different from cargo binding, such as activation for motility. The tail deletions (Figure 5) as well as the rest of the tail chimeras (Figure 6) are consistent with this notion.

However, the situation is more complex. If all elements of cargo binding and regulation were located in the tail, why should alterations in the neck/hinge domains compromise *in vivo* function? Motor activity is not affected in chimeras where these domains are exchanged (Grummt *et al.*, 1998b; this study). As shown in Figure 7, a chimera is fully functional in *Neurospora* if the neck, hinge, stalk–tail linker and tail, representing ~25% of the sequence, are derived from NcKin.

Two models can be envisaged to explain the requirement for endogenous neck and hinge regions. First, an unknown, species-specific regulatory molecule or complex might bind to these domains *in vivo* and regulate the activity of the motor. Secondly, kinesin could fold back upon itself to bring the tail portion of the molecule in contact with anterior domains, and this interaction requires compatible binding faces.

Two constructs both argue against the first and in favour of the second model. In NcΔ716–743 (Figure 7-10) and NcΔ551–634 (Figure 7-11), deletion of the stalk–tail linker and the kink, respectively, results in molecules with extended coiled-coils. Both constructs possess the endogenous tail required for cargo interaction as well as the endogenous neck and hinge. Even though they should be capable of binding to a putative regulator in the neck/hinge region, they rescue inefficiently, arguing against the first model. At the same time, they support the second model. The long coiled-coil and the lack of flexible domains would interfere with the ability of the molecule to fold, disrupting neck–tail interactions. We therefore consider the observations on the stalk chimeras to constitute strong evidence for the requirement for kinesin folding in the living cell. *In vitro*, kinesin was shown to exist in either an extended or more compact, folded configuration, depending on the ionic strength of the medium (Hackney *et al.*, 1992). Since the ATPase activity of full-length *Drosophila* KHC is suppressed, Stock *et al.* (1999) proposed that the tail domain folds back to inhibit the motor domain. The regions they identified to be important for intramolecular binding (the N-terminal part of the neck and the C-terminal part of the coiled-coil tail) can not be exchanged without a loss of function between SrKin and NcKin (Figures 6 and 7). In animal kinesins, the light chains are important for the inhibition of heavy chain binding to microtubules (Verhey *et al*., 1998), thereby indirectly supporting the folding model since a portion of the heavy chain tail that includes the light chain-binding site is needed for self-inhibition. Our findings extend the folding model to the *in vivo* situation and suggest the regions involved in intramolecular interaction are located in the neck/hinge region. To date, there is no evidence that self-inhibition involves the catalytic motor domain. The formal possibility that the tail interacts predominantly with a region in the head that is conserved between SrKin and NcKin is excluded by constructs Sr 751/740 Nc (Figure 6-10) and Sr 712/714 Nc (Figure 6-11). An inhibition of motor activity through an interference with the neck and hinge domains may seem surprising at first. However, evidence from several laboratories implicates both domains in important functions associated with mechanochemical transduction. The neck contributes to the coordination of the two heads in dimeric kinesin (Kozielski *et al.*, 1997; Thormählen *et al.*, 1998) and specifies the directionality of movement along microtubules (Case *et al.*, 1997; Henningsen and Schliwa, 1997; Endow and Waligora, 1998), while the hinge is required for the coupling of the ATPase activity and microtubule gliding velocity (Grummt *et al.*, 1998b). Thus the functional motor domain comprises not only the catalytic domain ('head') but also the adjacent neck and hinge domains. Interference with the neck/hinge region upon binding of the tail is therefore likely to affect motor function.

Another construct suggests that the *in vivo* function of kinesin is very sensitive to alterations in its geometry. In addition to the stalk–tail linker and kink deletions (Figure 7-10 and 7-11), the replacement of the NcKin stalk–tail linker by that of SrKin (Figure 7-9) also has a similar although less severe effect. This domain is unlikely to contain a crucial binding site as it is also exchanged in the construct shown in Figure 7-3 with no loss of function. The only difference is the length of the non-conserved portion of this domain (see Figure 4), which is 11 amino acids longer in SrKin. The increased length of the SrKin stalk–tail linker could lead to malpositioning of the C-terminus in the folded state or some form of steric hindrance that affects the molecular conformation. Interestingly, the effects of this replacement are alleviated if the kink and coil 2 are also changed to those of SrKin (Figure 7-3), suggesting that the combined kink–coil 2–stalk–tail linker segment of SrKin is compatible with the functionality of NcKin *in vivo*.

A final consideration concerns the existence of kinesin light chains in fungi. In animal species, kinesin light chains are apparently essential for kinesin function in organelle movements (Gindhart *et al.*, 1998). They may have a key role in the coupling of cargo association and motor function (Verhey *et al.*, 1998). Biochemical studies of all four fungal kinesins did not turn up any evidence for the existence of co-purifying light chains. Indeed, comparison of the light chain-binding site identified by Diefenbach *et al*. (1998) in animal kinesin (marked by the green dashed line in Figure 4) with the corresponding region in fungal kinesins shows a complete lack of homology. Thus, we consider it unlikely that light chains resembling those of animal species exist in fungi. Nevertheless, the segment that corresponds to the light chain-binding site in animal kinesins is conserved in all fungal kinesins, suggesting an as yet unknown but important function for this region. The sequence comparison (Figure 4) also brings to light a significant difference in domain topology between fungal and animal kinesins. In animal kinesins, the flexible region that marks the stalk–tail linker is located between the presumptive light chain-binding site and the conserved domain we identified as a site involved in cargo association. In fungal kinesins, however, the linker is shifted towards the N-terminus. Thus, the light chain-binding site of animal kinesins is part of coil 2, while the topologically equivalent domain of fungal kinesins is part of the coiled-coil tail. Interest-

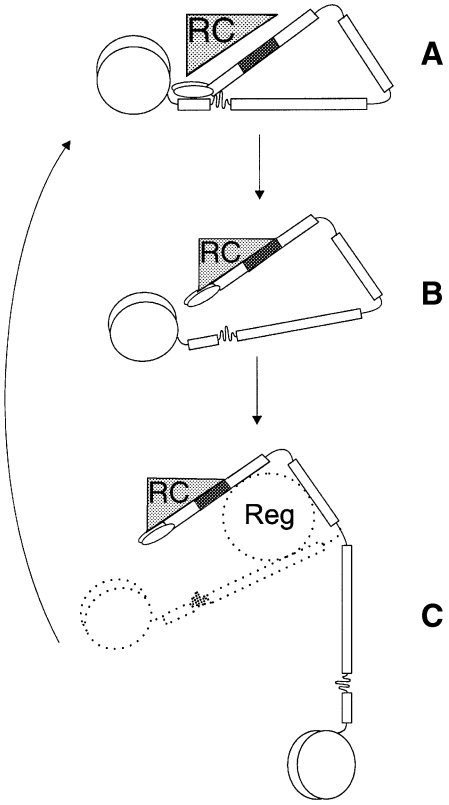

Fig. 8. A basic model for kinesin function. In (**A**), the closed, inactive state is depicted. RC represents the hypothetical receptor complex, and the cargo presumptive receptor-binding site is shown in dark gray. In (**B**), the receptor complex has bound to kinesin and the resulting conformational change leads to a derepression of the ATPase site in the kinesin functional motor domain. In (**C**), the loaded kinesin is active and transports its cargo. Upon cargo release, the molecule returns to state (A). The molecule may unfold completely, or it may be kept in a more folded configuration by additional regulatory factors (stippled model).

ingly, at the position of the stalk–tail linker in animals, fungal kinesins exhibit a discontinuity, or 'stutter', in the coiled-coil of the tail (marked with three asterisks in Figure 4). These differences in domain topology at the transition from the stalk to the tail may be of consequence for models of kinesin function in animals and fungi.

Consideration of all the features of the constructs presented here led us to propose the following model of the kinesin molecule *in vivo* (Figure 8). In this model, the coiled-coil domains are drawn approximately to scale. If a motif located C-terminally to residue 830 interacts primarily with the neck/hinge domains, as suggested by our data, the stalk would fold into a triangular shape. Such a configuration would be consistent with the importance of not only the flexible kink, but also the stalk–tail linker as a second flexible region, and would explain the dramatic effects caused by alterations in these domains. A cargo receptor (and regulatory) complex (RC) would associate with the putative cargo-binding region (marked dark gray) and would communicate cargo binding to the tail–neck/hinge interaction site, activating the motor for movement along microtubules. Activation could occur simply by releasing the neck/hinge from the tail, allowing the motor to unfold and interact productively with the microtubule surface. In the case of the kink and stalk–tail linker deletions, constitutive activation of kinesin would lead to an unbalancing of the kinesin regulatory system, thereby rendering the transport of the cargo less efficient. It is also conceivable that, in addition to the cargo receptor, there are as yet unknown regulator proteins that interact with NcKin and may keep it in a folded conformation even after activation.

In conclusion, the work presented here introduces a useful new assay to test the function of kinesin motors *in vivo*. It hints at the possible location of a site involved in cargo binding, and it extends and refines the folding model. With this assay at hand, we should now be in a position to pinpoint the sites involved in intra- and intermolecular interactions using point mutations in combination with localization studies.

Materials and methods

Vectors and strains

Escherichia coli strains MC 1061, XL 1 Blue and the *dam*$^-$ strain WA 321 were used for cloning. Mutant kinesins were cloned either into pSS5 or pCSN 43. pSS5 is a pBluescript vector containing the NcKin genomic DNA as an *Xba*I–*Eco*RI fragment and a Tn*5*-based bleomycin resistance cassette (Austin *et al.*, 1990) in the *Eco*RI site. A *Cla*I site was introduced immediately after the stop codon and used to insert C-terminally deleted fragments generated by PCR. pCSN 43 (Austin *et al.*, 1990) is a pBS-derived vector with the *Aspergillus nidulans* TrpC promoter and a *Cla*I site immediately before the start codon. It also contains the Tn*5*-based bleomycin resistance cassette downstream of the coding sequence.

For the chimeric and C-terminal deletion constructs, convenient restriction sites were introduced using PCR. The deletion constructs were generated by PCR with a 5′ primer that contained 18 bp upstream and downstream of the desired deletion. The PCR product was then used as primer in a second PCR as described by Landt *et al.* (1990). All preparative PCRs were carried out with the Boehringer Expand High Fidelity PCR System. The PCR-generated portions of all constructs were sequenced before transformation into the fungus. DNA sequences were obtained from TopLab (Munich) or MediGene (Munich).

Constructs were cloned into the NcKin null mutant generated from the *N.crassa* strain 74A (Seiler *et al.*, 1997). DNA-mediated transformation of *N.crassa* spheroblasts was carried out as described by Schweitzer *et al.* (1981), with modifications introduced by Akins and Lambowitz (1985). Briefly, polyethylene glycol (PEG)-competent spheroblasts were mixed with DNA and kept on ice for 30 min. After the addition of PEG, the mixture was then incubated for 20 min at room temperature and finally plated out in an agar containing 4 μg/ml bleomycin. Genomic DNA was isolated using the method described by Schechtmann (1986).

Growth and handling of fungal cultures

Neurospora crassa wild-type and mutant clones were grown in Vogel's minimal medium as described (Sebald *et al.*, 1979). Large cultures (>500 ml) were grown at 25°C under light and constant aeration. Cultures of 500 ml or less were grown in shaking culture at 25–30°C. The cultures were grown for 16–48 h, depending on the amount of conidia used for inoculation. General procedures used in the handling of *Neurospora* can be found in Davis and DeSerres (1970).

Characterization of fungal clones containing mutant kinesins

DNA taken up by *Neurospora* randomly integrates into the genome. Since not every bleomycin-resistant clone also expresses the transfected construct, resistant clones had to be checked for protein expression. As we were looking for rescue of the null mutant phenotype, any clone that appeared to be growing faster than the null mutant was of potential interest.

For each construct, at least 30 large, healthy clones were picked from the bleomycin-containing agar plate and put into a glass tube containing agar with a sloped surface. Clones whose pattern of growth after 24 h signalled that they were rescued at least partially were put on an agar plate and their growth speed was compared with that of a wild-type transformant. The fastest clones resulting from every mutant construct were selected.

From all mutant clones, the motor was isolated as described in Steinberg and Schliwa (1995) and its *in vitro* gliding velocity was measured using a standardized gliding assay (Steinberg and Schliwa, 1996) to determine whether mechanochemical activity was unaffected. The longitudinal growth rate of mutant clones was determined in a so-called race tube assay (Davis and DeSerres, 1970). A 30 cm long hollow glass tube of 1 cm diameter was half-filled with Vogel's minimal agar and inoculated with conidia at one end. Growth of the mycelium along the length of the glass cylinder was then recorded over time. With every clone transformed with a mutant construct, wild-type and null mutant cells were assayed in parallel under the same experimental conditions to assure comparability. The percentage of rescue was calculated as follows:

$$rc = [(grc - grn)/(grWT - grn)] \times 100$$

with rc being the percentage of rescue of the given clone, grc the growth rate of the clone, grWT the growth rate of the wild-type and grn the growth rate of the null mutant. The average of at least three race tube assays of the fastest growing clones was determined and rounded off to the nearest multiple of 5%.

If a given construct did not show any visible rescue, all bleomycin-resistant clones picked were grown in small-scale liquid culture, and the presence of expressed protein was determined by Western blot analysis (see below).

Determination of kinesin expression levels in positive clones

A cytoplasmic extract of 5 g of hyphae (wet weight) grown in liquid culture was prepared under standardized conditions as described (Steinberg and Schliwa, 1995) and centrifuged at 4°C and 140 000 *g* for 60 min in a Beckman ultracentrifuge. The supernatant was subjected to SDS–PAGE (Laemmli, 1970) using a 7.5% acrylamide gel. Western blotting was carried out according to Towbin *et al*. (1979).

In some experiments, kinesin was purified additionally by microtubule affinity as described in Steinberg and Schliwa (1995) and then subjected to Western blotting. The antibodies used were raised against full-length NcKin (Steinberg and Schliwa, 1995) as well as two different peptide motifs in the C-terminus (J.Kirchner, G.Steinberg and M.Schliwa, unpublished). In all experiments, wild-type kinesin prepared under identical conditions was used as a control. Western blots were quantified using the Stratagene EagleEye system and NIH Image.

We determined the protein expression levels of the C-terminal deletion constructs (see Figure 5; for construct nomenclature, see below). While constructs NcΔ923 (500%), NcΔ907 (110%), NcΔ859 (110%), NcΔ770 (140%) and NcΔ740 (150%) show at least a wild-type level of protein expression, the constructs NcΔ895 (35%), NcΔ830 (10%) and NcΔ801 (10%) exhibit a lower expression level (see Figure 5). However, protein expression levels do not correlate with the growth rates of these constructs. First, constructs NcΔ770 and NcΔ740 have a null mutant growth rate with a wild-type protein expression level. Secondly, constructs NcΔ923, NcΔ907 and NcΔ859 show intermediate growth rates with at least wild-type protein expression levels. Finally, construct NcΔ801 has a null mutant growth rate, while construct NcΔ830 which shows the same protein expression level, grows at an intermediate speed. Additionally, in transformations of wild-type and other fully rescuing constructs, we often obtained protein expression levels of 200–300% (data not shown), indicating that overexpression of fully functional kinesins has no effect on growth speed. Thus, protein expression levels seem to have a minor influence on the growth rate and are not rate-limiting. In the course of this work, we determined the protein expression levels of all relevant constructs (data not shown) and found our conclusions from the analysis of the deletions to be corroborated in all cases.

Construct nomenclature

C-terminal deletion mutants were named NcΔX, with X being the last amino acid present in the mutant. Internal deletion constructs were named NcΔX–Y, with X being the first and Y the last residue of the deletion. Chimeras were named according to the following scheme: initials of first species + number of last amino acid of this species at fusion site/number of first amino acid of second species at fusion site + initials of second species. Double chimeras follow the same pattern.

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