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Report

Neck Linker Length Determines the Degree of Processivity in Kinesin-1 and Kinesin-2 Motors

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Summary

Defining the mechanical and biochemical determinates of kinesin processivity is important for understanding how diverse kinesins are tuned for specific cellular functions. Because transmission of mechanical forces through the 14–18 amino acid neck linker domain underlies coordinated stepping [1–6], we investigated the role of neck linker length, charge, and structure in kinesin-1 and kinesin-2 motor behavior. For optimum comparison with kinesin-1, the KIF3A head and neck linker of kinesin-2 were fused to the kinesin-1 neck coil and rod. Extending the 14-residue kinesin-1 neck linker reduced processivity, and shortening the 17-residue kinesin-2 neck linker enhanced processivity. When a proline in the kinesin-2 neck linker was replaced, kinesin-1 and kinesin-2 run lengths scaled identically with neck linker length, despite moving at different speeds. In low-ionic-strength buffer, charge had a dominant effect on motor processivity, which resolves ongoing controversy regarding the effect of neck linker length on kinesin processivity [3, 5–7]. From stochastic simulations, the results are best explained by neck linker extension slowing strain-dependent detachment of the rear head along with diminishing strain-dependent inhibition of ATP binding. These results help delineate how interhead strain maximizes stepping and suggest that less processive kinesins are tuned to coordinate with other motors differently than the maximally processive kinesin-1.

Results and Discussion

Extending the Kinesin-1 Neck Linker Decreases Processivity

To investigate the influence of neck linker length on kinesin-1 processivity, we visualized homodimeric *Drosophila* conventional kinesin, truncated at residue 559 and fused to a C-terminal GFP, moving along immobilized bovine brain microtubules by single-molecule total internal reflection fluorescence (TIRF) microscopy. To minimize any electrostatic tethering between the motor and microtubule that can complicate the interpretation of mechanical processivity, we performed experiments in 80 mM PIPES buffer. The control Kin1 motor moved at 990 ± 130 nm/s (mean ± standard deviation), with a mean run length of 2.1 ± 0.1 μm (mean ± standard error of fit) (Figure 1A). When the neck linker domain was extended by three residues ($Kin1_{+DAL}$, corresponding to the last three residues of the kinesin-2 neck linker), the run length dropped by a factor of five to 0.39 ± 0.02 μm and the speed dropped to 600 ± 89 nm/s. Interestingly, inserting only one amino acid ($Kin1_{+L}$) diminished the run length to 0.89 ± 0.06 μm and the speed to 580 ± 95 nm/s (Figure 1B). Finally, shortening the neck linker by one residue ($Kin1_{\Delta T}$) abolished processivity (see Supplemental Results and Discussion and Figure S1 available online). These results confirm and extend previous work with full-length kinesin attached to beads [6] and demonstrate that kinesin-1 processivity is significantly reduced when the neck linker domain is extended by even one amino acid.

Shortening the Kinesin-2 Neck Linker Enhances Processivity

The intraflagellar transport motor kinesin-2 has a 17 amino acid neck linker, three residues longer than kinesin-1, and in previous work we showed that full-length kinesin-2 is 4-fold less processive than kinesin-1 [6]. If the reduced processivity of kinesin-2 results from diminished coordination between the heads that results from its longer neck linker domain, then a simple prediction is that shortening the kinesin-2 neck linker domain should enhance processivity. To test this, we made a motor consisting of the head and neck linker of the mouse KIF3A subunit of kinesin-2 (ending in the last residue of the neck linker domain, Leu359) fused to the neck coil and proximal rod of *Drosophila* kinesin-1 (starting at the first residue of the neck coil domain, Ala345) (Figure 1C). This kinesin-2 construct was used so that any differences in processivity can be attributed solely to the head and neck linker regions, and not to differences such as charge or mechanical integrity of the coiled coil. Whereas constructs containing the kinesin-2 coiled coil were only functional when they were baculovirus-expressed, these kinesin-1/kinesin-2 chimeras were functional when bacterially expressed and had similar properties to a baculovirus-expressed KIF3A homodimer investigated previously [6]. These GFP-tagged chimeric constructs are referred to as Kin2 throughout this paper.

The mean run length and speed of control Kin2 were 0.71 ± 0.03 μm and 480 ± 98 nm/s, respectively. To test whether shortening the neck linker enhances processivity, we made stepwise deletions of one, two, and three amino acids in the last three residues (DAL) in the kinesin-2 neck linker to create $Kin2_{\Delta A}$, $Kin2_{\Delta DA}$, and $Kin2_{\Delta DAL}$. Single-molecule run lengths and velocities were measured in a manner identical to Kin1. Deleting one residue ($Kin2_{\Delta A}$) increased the run length to 1.26 μm and had no effect on the velocity (484 nm/s), supporting the hypothesis. However, deleting two residues ($Kin2_{\Delta DA}$) decreased the mean run length to 0.59 μm, which is less than the control Kin2 motor. Deleting all three residues ($Kin2_{\Delta DAL}$) resulted in no observable processive runs in the single-molecule assay (Figure 2C), though the motors were functional in the multimotor gliding assay (Supplemental Results and Discussion and Table S2). Hence, although the results qualitatively agree with the hypothesis—shortening the kinesin-2 neck linker enhances processivity—there was not quantitative agreement between the kinesin-1 and kinesin-2 results.

Both KIF3A and KIF3B contain a proline residue at position 13 of the neck linker domain (Figure 1C), and in the only kinesin-2 crystal structure containing the entire neck linker domain (human KIF3B; Protein Data Bank ID code 3B6U), this proline is

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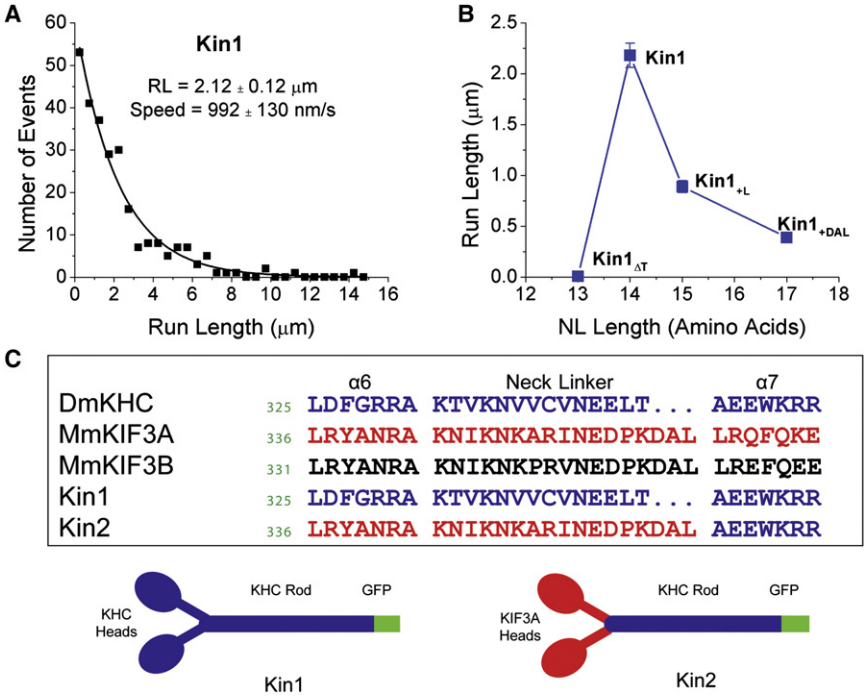

Figure 1. Kin1 Run Lengths and Design of Kin1 and Kin2 Constructs

(A) Run length of control Kin1 from total internal reflection fluorescence assay. Data were fit to a single exponential.

(B) Run lengths of different Kin1 constructs as a function of their neck linker (NL) length. Error bars represent the standard error from exponential fits.

(C) Amino acid sequence of the kinesin-1 (KHC) and kinesin-2 (KIF3A) neck linkers with the adjacent α6 (last helix in the head domain) and α7 (neck coiled-coil domain). The Kin1 construct includes the entire DmKHC sequence up to residue 559 (in the break between coil 1 and coil 2 of the rod domain [19]) followed by a C-terminal GFP and His_6 tag. The Kin2 construct includes the KIF3A head and 17-residue neck linker domain ending at Leu359 (red sequence) fused to the DmKHC neck coil and rod, starting at Ala345, the first residue in the neck coil domain (dark blue sequence). The cartoon shows the structures of the Kin1 and Kin2 constructs. The neck linker sequences for all constructs used are given in Table S1.

in the kinked *cis* conformation. We used molecular dynamics simulations to compare the predicted force-extension profiles of kinesin-1 and kinesin-2 neck linkers and found that, whereas the kinesin-1 neck linker was well fit by a model of a 14-residue worm-like chain, the 17-residue kinesin-2 neck linker was shorter than predicted by a worm-like chain model, except at high forces where the proline was forced into the straight *trans* conformation [8] (Figure 2A). When the proline at position 13 was changed to an alanine and the last three residues of the kinesin-2 neck linker were deleted, the modeled force-extension curve matched that of kinesin-1 (Figure 2B).

Kin1 and Kin2 Motor Properties Scale Similarly with Neck Linker Length

Motivated by these molecular dynamics simulations, we substituted the proline at position 13 of the Kin2 neck linker with alanine ($Kin2_{PA}$) and found that the mean run length fell from 0.71 μm to 0.39 μm, consistent with this substitution extending the effective neck linker length. Interestingly, the processivity of this $Kin2_{PA}$ construct closely matched that of the equivalent kinesin-1 construct, $Kin1_{+DAL}$ (0.39 μm) (Figure 2D; Table S2). More importantly, when the neck linker of this proline-substituted construct was shortened by three

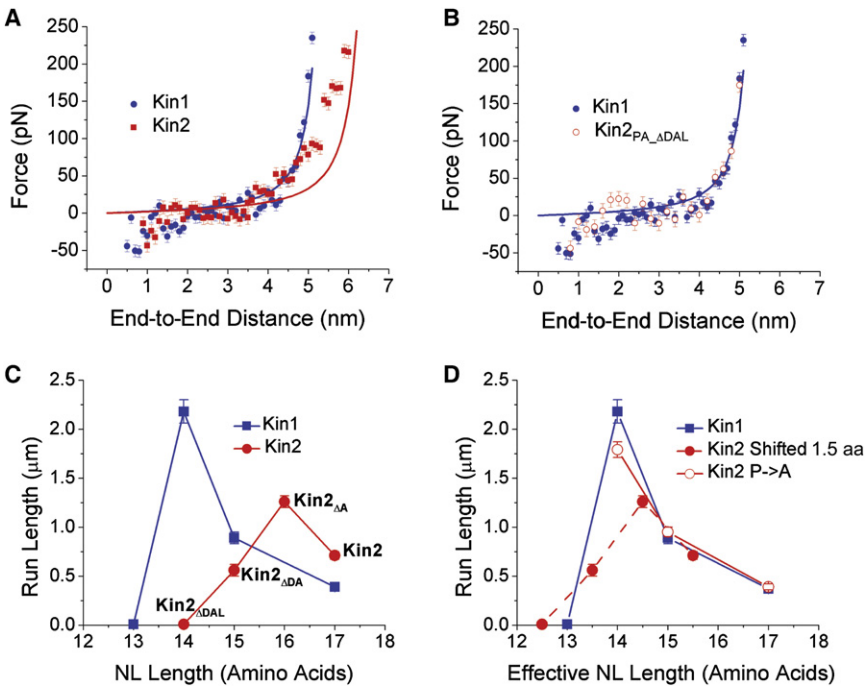

Figure 2. Kin1 and Kin2 Run Lengths Scale with Neck Linker Length

(A) Force-extension curves of kinesin-1 and kinesin-2 neck linkers from molecular dynamics simulations. Solid lines are predictions of a worm-like chain model for 14- and 17-residue polypeptides showing good fit for kinesin-1 (14 residues) and poor fit for kinesin-2 (17 residues). Data are replotted from [8].

(B) Predicted force-extension curve when the kinked proline in kinesin-2 is replaced with an alanine and the last three amino acids are deleted ($Kin2_{PA\_\Delta DAL}$), compared to kinesin-1.

(C) Comparison of run lengths for Kin2 and Kin1 constructs with identical neck linker lengths (number of amino acids).

(D) Run lengths following substitution of the *cis* proline in the Kin2 neck linker with alanine ($Kin2_{PA}$). $Kin2_{PA}$ constructs containing 14-, 15-, and 17-residue neck linkers are $Kin2_{PA\_\Delta DAL}$, $Kin2_{PA\_\Delta DA}$, and $Kin2_{PA}$, respectively. The curve for control Kin2 motors is shifted 1.5 amino acids to the left to account for the *cis* proline. All run length and velocity values are given in Table S2.

residues to match the length of the kinesin-1 neck linker, the mean run length of this Kin2$_{PA_\Delta DAL}$ construct rose to 1.8 μm, more than twice the native Kin2 processivity and very nearly matching the 2.1 μm run length of wild-type Kin1 (Figure 2D). To further examine this correlation, we then made an intermediate length construct, Kin2$_{PA_\Delta DA}$, containing a 15-residue neck linker domain and found that its run length closely matched the kinesin-1 construct having a 15-residue neck linker, Kin1$_{+L}$. The striking result here is that when the kinesin-2 neck linker is straightened by removing the *cis* proline, the run lengths of kinesin-1 and kinesin-2 motors match and scale similarly with neck linker length even though motor velocities remain distinct. As an example, the run lengths of Kin1 and Kin2$_{PA_\Delta DAL}$ are nearly identical, although their motor velocities differ by nearly a factor of two (990 ± 130 nm/s versus 508 ± 71 nm/s).

To extend this correlation of processivity with neck linker length, it is possible to estimate the degree to which the proline in the *cis* conformation shortens the kinesin-2 neck linker. Shortening the Kin2 neck linker by one residue increases processivity, whereas shortening it by two residues diminishes processivity and shortening it by three abolishes processivity altogether (Figure 2C). This is consistent with the maximum predicted run length lying between a deletion of one and two residues. When the Kin2 curve is shifted to the left by 1.5 amino acids, all three curves fall on top of one another (Figure 2D), suggesting that the kinked proline shortens the neck linker domain by the equivalent of 1.5 amino acids (~0.5 nm).

Adding Positive Charge in the Neck Linker Enhances Processivity

Although it is clear that neck linker length controls processivity, the degree to which charged residues in the neck linker also affect processivity is not clear. Positively charged residues in the neck coil domain and in the core head domain have been shown to enhance processivity through favorable electrostatic interactions with the microtubule [9, 10]. Understanding the dependence of kinesin processivity on neck linker charge and buffer ionic strength is important for properly interpreting the present data and for resolving disparate results in the literature. To test the extent to which the reduced run length of Kin1$_{+DAL}$ is due to unfavorable electrostatic interactions caused by the negative charge of the insert (−1 at physiological pH), we instead inserted a neutral three-residue insert, AAL. Kin1$_{+AAL}$ had a similar run length (0.45 μm; Figure 3A), confirming that the DAL insert reduces processivity by lengthening the neck linker and not by introducing negative charge. In contrast, extending the neck linker with a positively charged insert, KAL (Kin1$_{+KAL}$), resulted in a run length of 1.27 μm, which is more than 2-fold greater than Kin1$_{+DAL}$ or Kin1$_{+AAL}$ but is still considerably less processive than Kin1 (2.1 μm) (Figure 3A). Hence, even in 80 mM PIPES buffer, positively charged residues in the kinesin neck linker domain enhance processivity. However, in this case, the reduction in processivity due to lengthening the neck linker still clearly dominates over any electrostatic effects from the positively charged lysine.

If electrostatic interactions are playing a role in kinesin processivity, then the effect should be magnified in low-ionic-strength buffers where charge shielding is minimized. To test the effect of ionic strength on kinesin processivity, we measured run lengths of Kin1 and Kin1$_{+KAL}$ in 12 mM PIPES buffer and compared the values to run lengths in 80 mM PIPES buffer. In 12 mM PIPES, the Kin1 run length doubled to 4.2 μm and the Kin1$_{+KAL}$ run length increased 4-fold to 4.6 μm, such that the Kin1 and Kin1$_{+KAL}$ run lengths were nearly identical (Figure 3B). Hence, in low-ionic-strength buffers, the reduction in processivity resulting from extending the neck linker was almost perfectly matched by the enhancement in processivity resulting from the positively charged lysine in the insert.

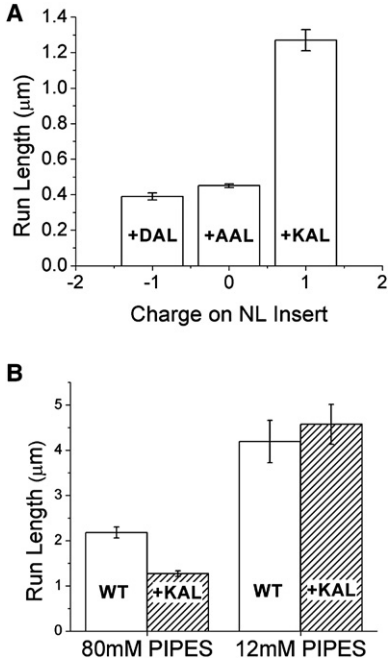

Figure 3. Neck Linker Positive Charge and Low Ionic Strength Enhance Processivity

(A) Kin1 run length as a function of the charge of the neck linker insert, showing that although negative charge does not diminish processivity, adding positive charge does enhance processivity. Experiments were carried out in 80 mM PIPES buffer.

(B) Effect of buffer ionic strength on control Kin1 and Kin1$_{+KAL}$ run lengths, showing that in 12 mM PIPES buffer, the diminished processivity due to the longer neck linker domain is compensated for by enhanced electrostatic interactions due to the added positive charge in the neck linker domain.

This finding that charge introduced in the neck linker plays a dominating role at low ionic strength helps to resolve the disparity between the present data and the results of Yildiz et al. [5], who found that inserts doubling the neck linker length had no significant effect on processivity. In that work, every insert in the neck linker contained an additional two lysines and a glycine; the authors argued that these positive charges compensated for moving the normal positive charge in the neck coil domain farther from the microtubule. Based on our results, the enhanced electrostatic interactions from these two lysines, which will be amplified in the 12 mM PIPES buffer used in that study, overwhelmed any reduction in processivity resulting from extending the neck linker domains. The simplest explanation is that these positively charged residues enhance processivity by interacting with the negatively charged C terminus of tubulin [9–11], although other mechanisms cannot be ruled out. We argue that for understanding the chemomechanical coordination between the two head domains that underlies kinesin processivity, these electrostatic effects should be minimized by using higher-ionic-strength buffers and minimizing positive charge in any sequence inserts.

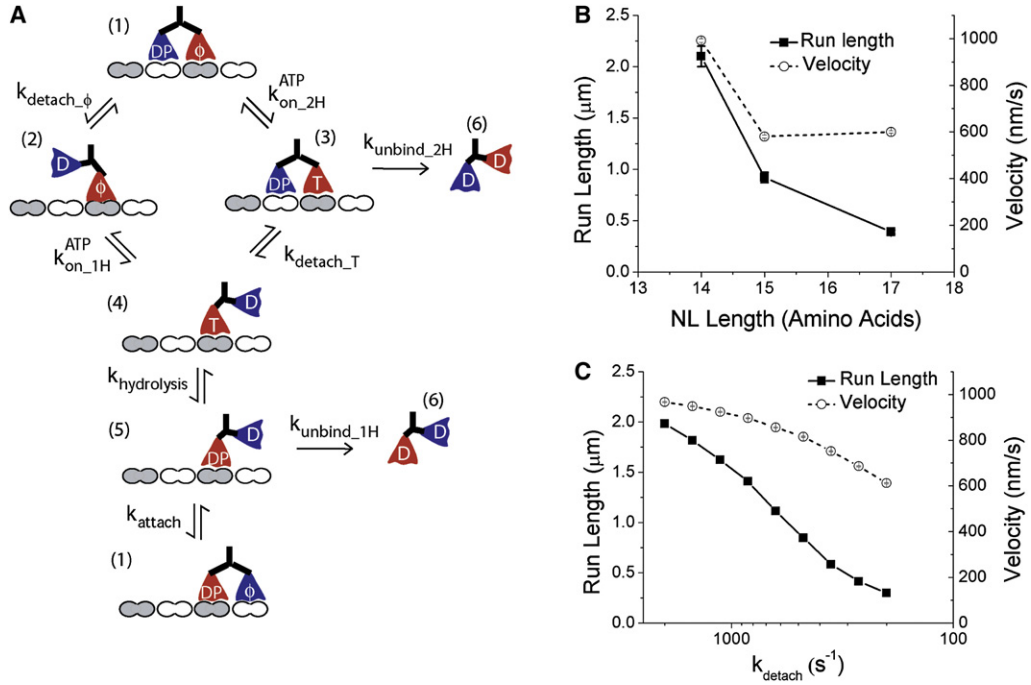

Figure 4. Modeling the Kinesin Chemomechanical Cycle

(A) Model for the kinesin chemomechanical cycle used to interpret the neck linker extension results. This framework is similar to a previous model [6], with the difference that motor unbinding from state 3 is combined into one rate constant (k_{unbind_2H}) for simplicity. Kinetic parameters are discussed in Supplemental Results and Discussion and listed in Table S3.

(B) Experimental Kin1 run length and velocity results, plotted as a function of neck linker length.

(C) Modeled run length and velocity from stochastic simulations of the model presented in (A), using rate constant parameters given in Table S3. In these simulations, $k_{on\_ATP\_2H}$ was set to 0.2 $\mu M^{-1}s^{-1}$ (10-fold above the best estimate from the literature [2]) and both k_{detach_φ} and k_{detach_T} were varied from 2000 s^{-1} down to 200 s^{-1} to model the effect of reduced strain on the trailing head due to extending the neck linker domain. Hence, for the model to account for the experimental results, extending the neck linker needs to alter two strain-dependent mechanisms—detachment of the trailing head and ATP binding to the leading head. Additional simulation results are given in Figure S4.

How Does Extending the Neck Linker Alter the Kinesin Chemomechanical Cycle?

To understand the mechanism by which extending the kinesin neck linker reduces processivity by a factor of five while reducing velocity less than 2-fold, we carried out stochastic simulations of the kinesin-1 kinetic cycle (Figure 4A) to identify whether modifications of individual model parameters are sufficient to account for the experimental results. Kinesin processivity is described by two mechanisms, front-head and rear-head gating [2, 4, 6]. Front-head gating holds that when both heads are bound (state 1), ATP binding to the leading head is inhibited by rearward strain, ensuring that the trailing head detaches before ATP binds (state 2). Rear-head gating holds that detachment from a one-head-bound state (state 5) is slow and forward-directed strain from the second head is necessary to detach the rear head (state 1 to 2 or state 3 to 4) at a rate consistent with the overall cycle time [4, 12].

For wild-type kinesin-1, where front-head gating is thought to block state 3 [2], processivity is determined by the relative rates of unbinding of the attached head (state 5 to state 6) versus rebinding of the tethered head (state 5 to state 1). To test whether changes in k_{attach} alone can account for the experimental results, we ran simulations at a range of k_{attach} values while holding all other parameters constant. Depending on the specific parameters used, the experimental results could be accounted for by positing that extending the neck linker reduces k_{attach} (Figure S4; Table S3). However, because attachment involves tethered diffusion of the unbound head, constrained by the entropic elasticity of the neck linker [6, 8, 13], extending the neck linker would be expected if anything to increase k_{attach} rather than decrease it. Nonetheless, this mechanism remains a formal possibility.

The next mechanism tested was the possibility that extending the kinesin-1 neck linker disrupts front-head gating by increasing the ATP binding rate in the two-head-bound state (state 1 to state 3 transition). To test whether a change in ATP binding alone is sufficient to account for the data, we varied $k_{on\_ATP\_2H}$ from 0.02 $\mu M^{-1}s^{-1}$ (consistent with experimental estimates in the strained state [2]) to 2 $\mu M^{-1}s^{-1}$ (the unstrained rate [2, 14]). A steep fall in run length was indeed observed, but velocity was unchanged (Figure S4). Hence, although this proposed mechanism can account for the effect of neck linker extension on run length, it cannot account for changes in velocity.

The third mechanism tested was the possibility that extending the neck linker domain slows strain-induced detachment of the trailing head. This strain-dependent detachment not only underlies rear-head gating, it also underlies front-head gating—detachment of the trailing head in state 1 must be very fast to prevent ATP binding to the front head and possible detachment from state 3. Depending on the rate constants chosen, decreasing the strain-induced detachment of the rear head (an expected outcome of lengthening the neck

linker) does result in a steep fall in run length and a moderate decrease in velocity (Figure 4C). However, this result is dependent on setting $k_{on\_ATP\_2H}$ to a value 10-fold faster than the experimentally estimated rate [2], setting k_{detach} to be very fast (2000 s^{-1}), and setting the unbinding rate from the two-head-bound state 3 to be 10-fold faster than the one-head-bound unbinding rate (state 5) (Table S3). Hence, changing either the ATP binding rate alone or the strain-dependent detachment rate of the trailing head alone fails to account for the experimental results. Instead, the neck linker extension results are best accounted for by both a slowing of the strain-dependent detachment rate k_{detach} and an increase in the strain-suppressed two-headed ATP binding rate $k_{on\_ATP\_2H}$.

The striking finding of this study is that virtually all of the difference in processivity between kinesin-1 and kinesin-2 motors results from differences in the length of the neck linker domain, and not from inherent differences in kinetic rates in the heads. Hence, when Kin1 and Kin2 neck linkers are identical lengths (following proline substitution), their run lengths match despite the fact that motor velocities differ by nearly a factor of two. The results can be accounted for by proposing that extending the neck linker both decreases strain-induced detachment of the trailing head (k_{detach}) and relieves the strain-inhibited binding of ATP to the leading head in the two-head-bound state. Because internal strain between the heads in kinesin-2 motors is less than in kinesin-1, it is expected that kinesin-2 responds differently to external strain such as during bidirectional transport of cargo or when many motors are cooperatively transporting cargo. Sequence predictions indicate that motors in different kinesin families have different neck linker lengths [8], and they are known to possess different degrees of processivity and work in diverse multi-motor arrangements; hence, this correlation of neck linker length with their cellular task may extend across the kinesin superfamily.

Experimental Procedures

Motor Constructs and Protein Expression

Kin1 was made by fusing *Drosophila* conventional kinesin truncated at position 559 to a C-terminal eGFP and His_6 tag. Kin2 was engineered by swapping the head and neck linker of mouse KIF3A into Kin1 (Figure 1C). See Supplemental Experimental Procedures for details on cloning procedures and sequences. All motors were expressed in bacteria and purified by Ni column chromatography as described previously [15, 16].

Motility Assays

Bovine brain tubulin was purified and labeled with Cy5 (GE Healthcare) as described previously [16–18]. Taxol-stabilized Cy5-labeled microtubules were adsorbed onto the surface of flow cells, and the surfaces were blocked with 2 mg/ml casein. Motility solution consisting of ~20 pM motors, 1 mM MgATP, 0.2 mg/ml casein, 10 μM Taxol, 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase, and 0.5% v/v β-mercaptoethanol in BRB80 (80 mM K-PIPES, 1 mM $MgCl_2$, 1 mM EGTA [pH 6.8]) was then introduced. Single-molecule run lengths were visualized by TIRF with a Nikon TE2000 microscope (60×, 1.45 NA Plan Apo) equipped with a 488 nm Ar ion laser for GFP excitation and a 633 nm He-Ne laser for Cy5 excitation; experiments were performed at 26°C. Images were captured with a Cascade 512 CCD camera (Roper Scientific), and acquisition and image analysis were carried out with MetaVue software (Molecular Devices); pixel size was 71.0 nm. The duration and distance of single motor runs were recorded manually. To ensure that all events were reliably captured, we only analyzed events with a minimum run length of 250 nm, and this minimum distance was subtracted from all runs (this assumes that detachment probability is independent of the distance that the motor has moved).

Supplemental Information

Supplemental Information includes Supplemental Results and Discussion, three tables, four figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.03.065.

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