UNDERSTANDING THE REGULATORY MECHANISMS OF MICROTUBULE BASED MOLECULAR MOTORS

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Abstract

Cytoskeleton and its associated many cytoplasmic proteins determines the cell shape, transport system, cell motility, and cell division. The transport system within the cell plays a key role in regulating these mechanisms. Short-range transport is primarily carried out by actin and its associated motor protein myosin. In contrast, long-range intracellular transport predominantly depends on microtubule and its associated motor proteins, kinesin and dynein. Motor proteins are mechanochemical ATPases that convert chemical energy to drive mechanical work. ATPase cycle is integral to molecular motors' processive motility to drive long-distance intracellular transport. Detailed studies on biochemical and biophysical properties of motor proteins have been done on kinesin-1, first among the kinesin superfamily to be discovered.

Kinesin-3 constitutes one of the large families among the kinesin superfamily that plays critical roles in cellular and physiological functions ranging from intracellular cargo transport to cell division to development. Defective motor functions implicated in many developmental, neurological and cancerous diseases. Kinesin-3 motors are further branched out to five subfamilies (KIF1, KIF13, KIF14, KIF16 and KIF28) endowed with unique features and functions. Kinesin-3 motors are remarkably superprocessive, hence the name 'marathon runners' with high velocity, superprocessive motility and strong microtubule affinity. Previous studies from kinesin-1 and myosin motors shows that adenosine triphosphate (ATP) turnover rate regulates the stepping rate and processivity of the molecular motors. However, the regulating mechanism of this ATP turnover regulatory mechanism or the chemomechanics of kinesin-3 motors is poorly understood.

To understand the evolutionary modifications that furnished superprocessive nature to kinesin-3 motors and their regulatory mechanisms we employ cellular, biochemical and biophysical approaches. Such studies demand purified proteins on a large scale. Expression and purification of these motors using mammalian cells yield much less protein and these protocols would be expensive and time-consuming. In comparison, expressing these proteins in prokaryotic expression system resulted in significantly

inactive and protein aggregation. To overcome the limitations posed by bacterial purification systems and mammalian cell lysate, we have established a robust Sf9-baculovirus expression system to express and purify full-length and constitutively active kinesin-3 motors.

In the present work, we have established the purification of kinesin-3 motors using Sf9-baculovirus expression system. The full-length and constitutively active kinesin-3 motors are C-terminally tagged with 3-tandem fluorescent proteins that provide enhanced signals and decreased photobleaching, critical for single-molecule motility assays. First, we performed *in vitro* single-molecule and multi-motor gliding assays under Total Internal Reflection Fluorescence (TIRF) illumination using Sf9 purified proteins. We demonstrate that the functional output of motor proteins purified from the Sf9-baculovirus system are comparable to those expressed in mammalian cells. Remarkably, their motility properties are identical to that of motors prepared from mammalian cell lysates.

As the processivity is guided by ATP turnover rate by the motor proteins, we performed microtubule-stimulated ATPase measurements using Sf9 purified kinesin-3 motors. To do this, we adapted a colorimetric ATPase assay based on formation of phosphomolybdate complex formation. We show for the first time that kinesin-3 motors are robust ATPases with high ATP turnover rates, which is 1.3 to 3-fold higher compared to a well-studied kinesin-1 motor. Remarkably, these ATPase rates correlate to kinesin-3 motors stepping rate, suggesting a tight coupling between chemical and mechanical cycles.

Intriguingly, kinesin-3 velocities (KIF1A > KIF13A > KIF13B > KIF16B) show an inverse correlation with their microtubule-binding affinities (KIF1A < KIF13A < KIF13B < KIF16B). Interestingly, our *in silico* analysis using the coupled Brownian motor model also predicted a similar trend (KIF1A > KIF13 > KIF16B) that is observed experimentally. We did mutational studies to demonstrate that the positively charged residues in loop8 of the kinesin-3 motor domain largely contribute this differential microtubule-binding affinity. Most importantly, the presence of arginine residue enables

multiple stable electrostatic interactions with negatively charged glutamate residue at the C-terminal tail region in tubulin. Collectively, we demonstrate that a fine balance between ATP hydrolysis and microtubule-binding affinity rate enables kinesin-3 motors with novel mechanical outputs.

To understand the collective behavior of kinesin-3 motors we performed microtubule-gliding assay with constitutively active kinesin-3 motors. Microtubule gliding with kinesin-3 motors displayed significant microtubule bending without affecting their gliding velocities compared a well-characterized motor, kinesin-1. Furthermore, we did *in vivo* cell culture studies to investigate the underlying mechanism of microtubule bending. Microtubule-bending analysis of cells expressing kinesin-3 motors showed significant bending compared to kinesin-1 motor. Our previous work has suggested the K-loop, a conserved; hallmark insert in loop 12 of kinesin-3 motor domain plays an essential role in motor microtubule interaction in the ADP state. Hence to dissect its role in bending we created K-loop mutations and performed live cell studies and *in vitro* microtubule gliding assay. Analysis from these studies suggests that positively charged lysine residues in the K-loop influence kinesin-3 driven microtubule bending in cells.

Together, we successfully purified full-length and constitutively active kinein-3 motors from Sf9-baculovirus expression system and performed biochemical and biophysical studies. We show that these purified proteins are of high quality and functionally on par with motors from mammalian lysates as demonstrated in single-molecule, gliding and ATPase assays. We also show that Loop8 of kinesin-3 motor domain largely contributes to the differential microtubule-binding affinities. Interestingly, these microtubule-binding affinities inversely correlate to the velocities of kinesin-3 motors. Furthermore, the K-loop, a positively charged insert in the loop12 of the kinesin-3 motor domain promotes microtubule-bending. Hence, we propose that kinesin-3 motors are fine-tuned at the molecular level to endow diverse mechanical outputs critical for myriad of cellular processes.