Suppressor analysis of the MotB(D33E) mutation to probe the bacterial flagellar motor dynamics coupled with proton translocation

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MotA and MotB form the stator of the proton-driven bacterial flagellar motor, which conducts protons and couples proton flow to motor rotation. Asp-33 of *Salmonella* MotB, which is a putative proton-binding site, is critical for torque generation. However, the mechanism of energy coupling remains unknown. Here, we carried out genetic and motility analysis of a slow motile *motB*(D33E) mutant and its pseudorevertants. We first confirmed that the poor motility of the *motB*(D33E) mutant is neither due to protein instability, mislocalization nor impaired interaction with MotA. We isolated 17 pseudorevertants and identified the suppressor mutations in the transmembrane helices TM2 and TM3 of MotA and in TM and the periplasmic domain of MotB. The stall torque produced by the *motB*(D33E) mutant motor was about half of the wild-type while it was recovered nearly to the wild-type levels for the pseudorevertants. However, their high-speed rotation under low load was still significantly impaired, suggesting that the maximal rate of proton translocation is still severely limited. These results suggest that the second-site mutations recover the rate of torque generation step by the stator-rotor interactions coupled with protonation/deprotonation of Glu-33 but not the reduced proton conductivity by the D33E mutation.

IP-153 ネックリンカーが正常にドッキングできないヘテロ二量体キネシンの連続歩行運動

Processive motility of heterodimeric kinesin that has defect in neck linker docking
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Kinesin is a motor protein that moves processively along microtubules in a hand-over-hand manner. The neck linker, a short stretch that connects two motor heads, has been shown to undergo ATP-dependent conformational changes, although its role on the processive motility is still controversial. We recently reported that heterodimeric kinesin that composed of wild-type head and a mutant R203K head that can normally bind ATP but is unable to hydrolyse ATP showed slow processive movement along microtubules and that in the rate limiting step the heterodimer took two-head-bound state where the R203K mutant head is in the rear. In this study, we observed conformational changes of the neck linkers of this heterodimer using single molecule FRET to investigate the role of the neck linker on processive motility of this heterodimer. First we observed the neck linker of monomeric R203K bound to the microtubule in the presence of ATP and found that the neck linker remained undocked and cannot stably take docked conformation. Next we observed the neck linker of the heterodimer during processive movement. The neck linker in the wild-type head showed normal undock-to-dock conformational changes, but the neck linker in the R203K head fluctuated and stayed mostly in intermediate FRET states. These results suggest that the neck linker docking in the microtubule-bound head is not required for the tethered wild-type head to bind to the forward tubulin-binding site and rather is required for promoting the detachment of the trailing head from microtubule.

IP-154 ミオシンVの足首の動きを見る（アクチンなし）

Watching 'ankle' action of myosin V in the absence of actin

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Myosin V is an actin-based linear molecular motor that 'walks' in discrete ~ 35-nm steps following a "hand over hand", alternating site mechanism in which the two feet ('heads' or 'motor domains') switch between leading and trailing positions along the actin. Previously, we have reported that the lifted foot has access to a next forward landing site by combination of its rotational Brownian motion through a flexible joint at the leg ('neck' or 'lever arm')-leg junction and forward movement of the joint by lever action of the landed leg. Our purpose here is to understand how the lifted foot binds to the leading site on actin. To step successfully, the actin binding site of the lifted foot should be properly oriented relative to actin, which largely depends on the ankle angle so that the 'toe' of the lifted foot points down towards the actin.

To observe and characterize ankle action in the absence of actin, we fixed the leg of monomeric myosin V on a substrate, attached beads to the foot and visualized the ATP-dependence of bead position fluctuations. When triggered by UV flash of caged ATP, duplex beads swing 60-100 degrees, maintain this position for tens of seconds, then relax back to the original angle position. This cycle was observed repeatedly and only by UV flash of caged ATP. To characterize its dependence on ATP concentrations qualitatively, we are estimating ATP concentrations by measuring rotational speed of rotary motor, F₁-ATPase. We will discuss the ankle action for the lifted foot landing on a forward site.

IP-155 レーザー照射により励起された微小管のバイアスブラウン運動 Biased-Brownian-motion of microtubules energized by laser irradiations

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Many types of biological motion were supported by filamentous rail protein and ATP hydrolyzing motor proteins. By the result of the recent study, detailed

molecular mechanism of motor proteins has been clarified. On the contrary, knowledge about how the energy of ATP was used to actuate a motor protein. According to the thermal ratchet model, temperature difference between motor and rail proteins might be final figure of the actuating energy.

On this point of view, we have developed an artificial moving system of an actin filament equipped with gold particles with accepting energy of infrared laser. The actin filament of this system displayed unidirectional motion with an instantaneous velocity of about 5.0 micrometer/sec. However, the motion was intermittent and the difference between thermal Brownian motion was not so remarkable. In order to conquer the problem, we have used microtubules as a fabulous protein. As a energy acceptor, we intended to approach in two methods. Gold particle with the size of 1.4 nm in aqueous solution was reported to absorb laser energy only 1.0 or less. By introducing microtubules, the absorbing efficiency was expected to increase up to 10. Next method is to use a semiconductor particle, so-called Q-dot, with a diameter of about 10 nm as a laser acceptor.

Now, we have prepared microtubules decorated with secondary antibodies associating Hilyte Fluor555 dye. Artificial movement of microtubules would be shown at the meeting.

IP-156 ストックヘッドの変異による細胞質ダイニンの連続運動性の人為的操作

Engineering the processivity of cytoplasmic dynein

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Cytoplasmic dynein is a two-headed processive motor responsible for diverse intracellular motility. How it remains attached to the microtubule track through multiple ATPase cycles is still a mystery. In the present study, we assessed the structural features that affect the processive run length by analyzing the properties of a chimera containing the poorly processive Dictyostelium cytoplasmic dynein motor domain and the human axonemal dynein 7 microtubule-binding domain (stalk head). The chimera showed longer run lengths than the wild-type Dictyostelium cytoplasmic dynein construct, suggesting that the higher affinity for microtubules made the motor more processive. We are now analyzing the processive behavior of the chimera in more detail.

IP-157 ゲルゾリン C 末端部位を用いたアクチン精製法

Purification of actin by rapid affinity chromatography using C-terminal half segment of gelsolin

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In living cells actin filaments undergo continuous, dynamic turnover and remodeling. These processes involve polymerization, depolymerization, severing, capping, and branching of actin filaments through interaction with the actin-binding proteins. Gelsolin is one of these proteins, which regulates the state of intracellular actin by severing the actin filament at substoichiometric concentrations and by capping the barbed end of actin filaments. Here we describe a rapid and mild affinity-purification method using C-terminal segment of gelsolin (G4-G6) for the isolation of actin from mammalian cells. Recombinant actin obtained in the baculovirus expression system was also purified by G4-G6 affinity chromatography. Unlike DNase I-actin complex, G4-G6 dissociated from actin simply by removing Ca²⁺ from the solution without the need to use a denaturant, such as formamide and guanidine hydrochloride. His-tagged G4-G6 was expressed in *Escherichia coli* and formed a complex with beta-actin in the presence of Ca²⁺, then bound to Ni-sepharose beads (GE Healthcare). Purified actin was obtained by washing actin/G4-G6/Ni-sepharose beads with EGTA, followed by G-F transformation. At this meeting we report in detail the purification method and the properties of the purified actins.

IP-158 キネシンの連続歩行には適切な空間配置が重要である

DNA-kinesin Hybrid-nanomachine reveals that optimized layout is crucial for kinesin's processive run

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The motor protein kinesin has two heads (catalytic domains) and walks along microtubule, using the energy of ATP. It has been proposed that the space layout of the two heads is important for the processive movement. However precise mechanism has not been elucidated yet. Here we create hybrid-nanomachine composed of DNA and kinesin motor protein (DNA-kinesin), using DNA as a skeletal structure and protein as a functional module. Single molecule imaging of DNA-kinesin allows us to evaluate the effect of connect position (N, C-terminal or mid position) and distance between two heads with sub-nanometer step interval. Our results show that the native C-terminal connection and the distance between two heads is not essential for the processive movement, but is for the most an efficient layout. These results suggest that connection with appropriate distance of two heads through the neck linker, a 14 amino acid chain positioned at the C-terminal part of the head, is the key difference in function between monomer and dimer. Thus, dual function of the neck linker (swinging tethered head forward by conformational change at a one-head bound state, and coordinating two heads through the internal load at two-head bound state) ensures the kinesin's efficient processive movement.