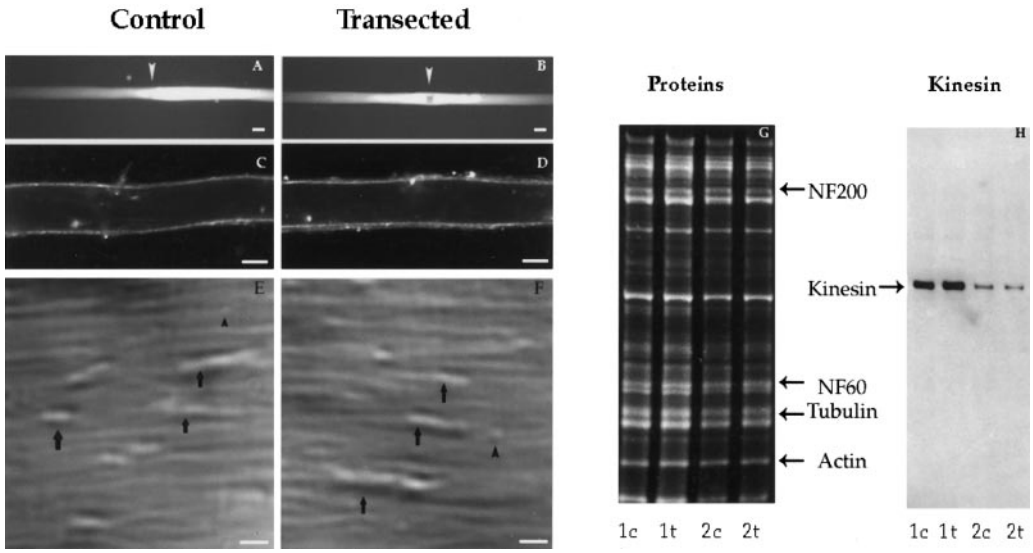

Figure 1. *Effects of axotomy on axonal transport, structure and proteins. Fluorescent tubulin injected into an axon moved anterogradely (to the right) away from the injection site (arrowhead in A). Tubulin injected into an axon that had been separated from its cell bodies 2 d prior showed no anterograde transport (B). This tubulin just diffused in both directions around the injection site (arrowhead in B). Darkfield light scattering was unaffected by axotomy. Light scattering was the same in control (C) as it was in transected axons (D). Higher resolution video enhanced differential interference microscopy also showed no difference between control (E) and paired transected (F) axons. Mitochondria (arrows in E and F) and smaller organelles (arrowheads) were present in control and transected axons. Scale bars: 100 μm for A and B, 50 μm for C and D, and 1 μm for E and F. Solubilized axonal proteins (G) from equal lengths of axon from the control (1c) and the transected side (1t) of a squid that had been axotomized 2 d earlier showed no change in protein-staining intensity. Paired axons from a smaller squid (2) with shorter axons had proportionately less protein than the larger squid (1), but there was no significant difference in protein levels between the control side (2c) and the transected side (2t). Similarly, axotomy had no effect on total immunochemically measured kinesin levels (H) in the paired axons from these same two squids. (1c* vs. *1t and 2c* vs. *2t).*

The present experiment suggests that some unidentified factor essential to axonal tubulin transport is lost or inhibited 2 days after axotomy. This inhibition is not due to the depletion of conventional kinesin. It could be due to the inactivation of conventional kinesin or to the inactivation or depletion of some other motor or factor essential to axonal tubulin transport. This model of protein transport in the squid giant axon may facilitate the identification of some of the factors essential to the control and maintenance of slow axonal transport.

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Interactions Between Recombinant Conventional Squid Kinesin and Native Myosin-V

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Axoplasm from the squid giant axon is one of a small number of cell-free extracts within which axonal transport can be studied *in vitro* (1). In squid axoplasm, one can observe both microtubule-based and actin-based vesicle transport and the seamless transition of vesicles from microtubules to actin filaments (2). Based on studies of vesicle transport in this cell-free preparation, a new model of axonal transport has emerged called "dual transport" in which long-range vesicle transport is microtubule-based while short-range transport is actin-based (3).

An exciting recent discovery is the finding that the cargo-binding domains of myosin-V, an actin-based motor, and kinesin, a microtubule-based motor, interact to form a hetero-motor com-

plex (4, 5, 6, 7). The interaction of myosin-V with kinesin has been established through yeast 2-hybrid assay, co-immunoprecipitation, co-affinity isolation, and co-purification with myosin-V (4, 5, 6, 7). The distal/globular tail domain of myosin-V binds to the rod-tail domain of kinesin in the "hetero-motor" complex. The members of the kinesin super family that have been shown to bind to myosin-V include conventional or ubiquitous kinesin (kinI) and Smy1p (4, 5, 6, 7). Evidence that myosin-V and kinesin interact on the membrane surface has not yet been demonstrated.

In this report, we performed experiments to determine the functional significance of the interactions between kinesin and myosin-V. Our working hypothesis is that tail-tail interactions between these motors provide feedback and thereby allow coordination of motor activity during the transition of vesicles from microtubules to actin filaments. For example, one motor may become inactive when its partner is actively transporting a vesicle along a filament (3). Several studies have shown that the ATPase activity of kinesin is inhibited when the head and tail domains of the molecule interact (8, 9). Auto-inhibition may be the mechanism by which one motor becomes inactive when its partner motor binds to a filament. Therefore, only one motor is actively engaged in movement and a tug-of-war between motors is prevented. Such feedback between motors could explain the seamless transition of vesicles from microtubules to actin filaments observed in the squid giant axon.

In this study we used a histidine-tag bound to the tail fragment of squid conventional kinesin (His-tagged) to study the interactions between kinesin and myosin-V. A cDNA construct coding for the rod-tail domain of conventional squid kinesin (SK KhcU; gift of K. Kosik) was engineered into a vector containing a His-tag. The 1.5 kb SK KhcU contained most of the rod II domain and the entire tail domain including the stop codon. The sequence of the insert was confirmed by PCR. The His-kinesin vector was expressed in *E. coli,* and the recombinant protein was then purified on a nickel-column. A gel of the fraction eluted from the column with 40 m*M* imidazole showed a prominent band at 45 kDa, the expected molecular weight of the fragment (Fig. 1A, lane 1). This band was identified as the His-labeled kinesin fragment on nitrocellulose membranes that were probed with the His-antibody (Fig. 1A, lane 2).

Next, we applied a clarified extract of His-kinesin tail fragment to a Ni-column to make a kinesin tail affinity column. Nonspecific proteins were removed by several buffer washes; and a clarified squid optic lobe extract was then passed over the column, followed by buffer washes. The proteins that bound specifically to the tail domain of kinesin were eluted with imidazole and analyzed by SDS-PAGE. Multiple protein bands were visible on the gel of the eluted fraction (Fig. 1B, lane 1). The proteins were transferred to membrane and probed with an antibody to myosin-V, a known binding partner of kinesin. A prominent band was observed on the blot (Fig. 1B, lane 2). These data showed that the recombinant His-kinesin fragment interacted with myosin-V and several other proteins in the squid brain extract. Finally, we asked whether the His-kinesin fragment blocked microtubule-based vesicle movement in motility assays. Preliminary experiments were performed in which the His-kinesin tail fragment was added to extruded axoplasm from the squid giant axon (Fig. 1C). Vesicle transport was measured by counting the number of vesicles moving/microtubule/min (v/mt/m; motile activity) at 15-min intervals. A reduction of motile activity occurred but the number of replicates was not sufficient to provide conclusive evidence.

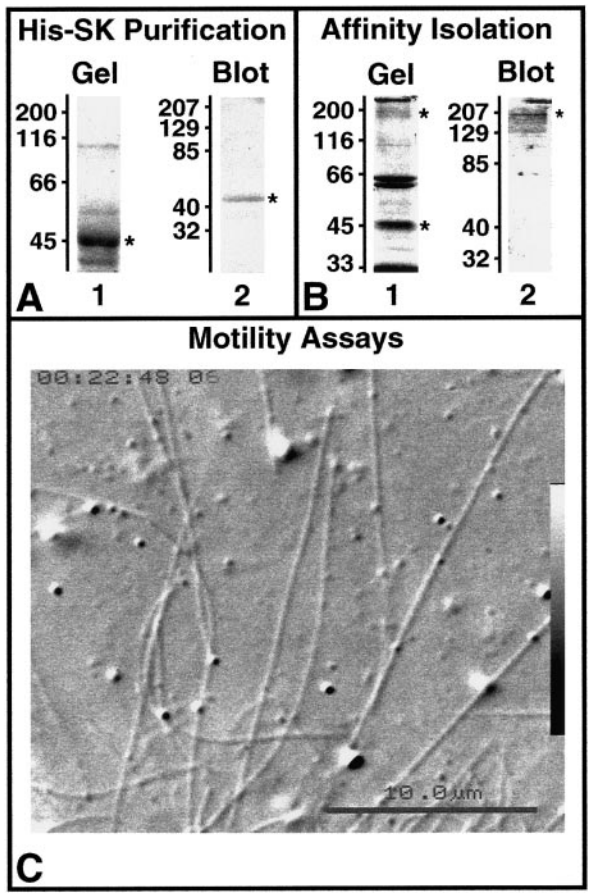

Figure 1. *(A) The presence of the His-tagged recombinant squid kinesin tail construct was verified using SDS-PAGE and western blots, and sequencing data. Lane 1 shows an SDS-PAGE gel confirming the presence of a protein with a molecular weight of 45 kDa, which matches the expected weight of the kinesin construct. Lane 2 shows a western blot probed with anti-His antibody; the blot confirms that the 45 kDa protein contains a His-tag. (B) The squid kinesin tail fragment is shown to interact with native squid myosin-V obtained from homogenized optic lobes. The homogenized extract was run through a His-kinesin affinity column. Lane 1 shows an SDS-PAGE gel of the elution fraction and shows that the 45 kDa kinesin tail and the 196 kDa native myosin-V are present. The elution fraction was blotted and probed with myosin-V antibody α-QLLQ (lane 2) and confirms myosin-V. (C) Allen Video Enhanced Contrast-Digital Interference Contrast (VEC-DIC) microscopy is used to image microtubules and vesicle motility in extruded squid axoplasm. Experiments are being performed to determine whether the recombinant kinesin tail fragment conclusively inhibits vesicle motility along microtubules.*

In summary, recombinant, His-labeled, squid kinesin tail fragment binds to squid brain myosin-V, as demonstrated by affinity column isolation. The recombinant tail fragment is thus an excellent tool for identifying specific binding partners of kinesin and potentially for studies of kinesin-mediated vesicle transport.

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Rab-GDI Inhibits Myosin V-Dependent Vesicle Transport in Squid Giant Axon

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Myosin-V, an actin-based motor, and kinesin, a microtubule-based motor, interact to form a "hetero-motor" complex (1). Axoplasmic vesicles containing these hetero-motor complexes move on microtubules in the axon and on actin filaments at the cell cortex (2). Negative feedback between these two motors is thought to facilitate the transition of vesicles from microtubules to actin filaments (3). Proteins that couple the hetero-motor complex to vesicles have not been identified. Recent studies have shown that the Rab family of GTPases is involved in the recruitment of myosin-V to vesicles. In melanocytes, myosin Va is recruited to melanosomes by Rab 27a (4, 5, 6). Melanophilin, an activator of Rab 27a, has been shown to be required for the binding of myosin-V to Rab 27a (7). Therefore, Rab 27a and melanophilin have been identified as the receptor complex for myosin Va on melanosomes. The Rab GTPase responsible for myosin-V recruitment to axoplasmic vesicles in the squid giant axon has not been determined, although Rab 3a is known to be associated with synaptic vesicles in squid brain (8). In this study we show that Rab-GDI pulled down myosin-V by affinity isolation and blocked vesicle transport in motility assays. We also show that the tail domain of myosin-V binds to tubulin dimers, presumably through kinesin or another linker protein.

To identify the Rab GTPase involved in myosin-V/kinesin binding to axoplasmic vesicles, and to identify all binding partners of the hetero-motor complex, we used a recombinant glutathione S-transferase (GST)-labeled globular tail domain of myosin-V (GST-GTD) and a GST-labeled Rab GDP dissociation inhibitor (GST-GDI) in motility and affinity isolation experiments. In a previous study, we showed that GST-GTD binds to squid brain vesicles and displaces the native myosin-V. The displacement of native myosin-V blocked transport of axoplasmic vesicles on actin filaments in assays using the squid giant axon (9, 10). In this study, the GST-GTD and GST-GDI were used to pull down binding partners of myosin-V for analysis by 2-D gel electrophoresis and protein sequencing.

A plasmid containing a cDNA insert for GST-mouse myosin-V AF6/Cno tail-globular-domain (gift from Dr. Huang) was expressed in *E. coli* (9). The bacterially expressed 84 kDa GST-myosin-V globular tail fragment was bound to a GST-column to generate an affinity column for isolation of binding partners of the myosin-V tail. Clarified squid brain extract was applied to the column and, after extensive washes, GST-GTD was eluted with glutathione. As a control, clarified brain extract was also applied to and eluted from a column without GST-GTD. GST-GTD was present in the eluted fraction from the GST-GTD column, as revealed by SDS-PAGE (Fig. 1A, lane 1). There were no proteins in the fraction eluted from the column in the absence of GST-GTD (Fig. 1A, lane 2). The presence of GST-GTD in the eluted fraction was confirmed by probing blots of this fraction with GST antibody (Fig. 1A, lane 3). Kinesin, a known binding partner of myosin-V, was identified in the fraction eluted from the GST-GTD column (Fig. 1A, lane 4). This fraction was analyzed further by 2-D gel electrophoresis. A pH range of 3–10 was used in the first dimension and an 8.5% SDS-PAGE gel for the second dimension. The 2-D gel was electroblotted to PDVF membrane (ProBlott, Applied Biosystems), and the protein spots were stained with Coomassie Blue R-250. Approximately 35 spots were clearly visible on the membranes (Fig. 1B). The major spots were excised and the N-terminal sequences were determined using an Applied Biosystem Procise sequenator. The identification of the proteins was based on sequence homology using BLAST.

Sequence information has been obtained for eight proteins excised from the 2-D gel. The two most interesting proteins thus far identified are α- and β-tubulin. These data show that tubulin dimers are retained on the column, suggesting that the tail domain of myosin-V binds directly or indirectly to microtubules. An indirect link between myosin-V and tubulin may be through kinesin, which binds directly to the tail of myosin-V (Fig. 1A, lane 4). Several studies have shown interactions between myosin-V tail and other proteins that bind to microtubules. Therefore, we conclude that tubulin binds to myosin-V indirectly, either through kinesin or another microtubule-associated protein.

A plasmid containing the full-length cDNA for *Drosophila* GST-Rab-GDI (gift from Dr. C. Cheney) was expressed in *E. coli.* The 75 kDa GST-tagged protein was purified on a GST affinity column (Fig. 1C, lane 1) and used in pull-down experiments with squid brain extracts. Myosin-V was identified as one of the proteins in the fraction eluted from the GST affinity column (Fig. 1C,