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Reference: *Biol. Bull.* **205**: 190–191. (October 2003)
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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,

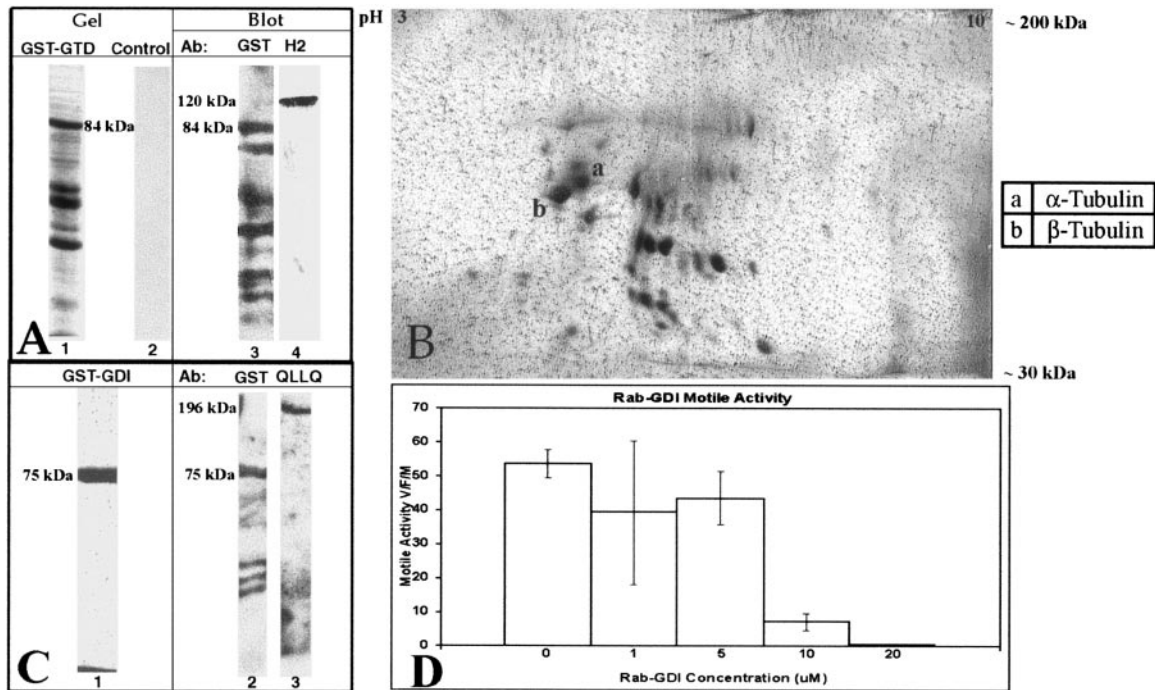


Figure 1. A plasmid containing GST-myosin-V tail (GST-GTD) was expressed in *E. coli*. The 84 kDa fusion protein was bound to a GST-affinity column, then clarified squid brain extract was applied to the column, washed, and eluted with glutathione to identify binding partners of the myosin-V tail. (A) SDS-PAGE gel of the fraction containing GST-GTD is shown in lane 1. A band at 84 kDa indicates the presence of GST-GTD. The lower molecular weight proteins are putative myosin-V tail-binding partners. An SDS-PAGE gel of the fraction eluted with glutathione from the control column (no GST-GTD) is shown in lane 2. There were no proteins in this fraction. A blot of the GST-GTD fraction probed with GST antibody (lane 3) confirmed the presence of the myosin-V tail fragment (84 kDa). The lower molecular weight bands represent breakdown products of the GST-GTD fusion protein. A blot of the GST-GTD fraction probed with H2 (lane 4) shows the presence of kinesin (120 kDa), indicating interaction between myosin-V and kinesin. (B) A 2-D gel of the GST-GTD fraction shows approximately 35 spots. Two identified spots, a and b, are α -tubulin and β -tubulin, respectively. (C) A plasmid containing GST-Rab-GDI (GST-GDI) was expressed in *E. coli*, applied to a GST affinity column, and eluted with glutathione. An SDS-PAGE gel of the fraction containing GST-GDI (75 kDa) is shown in lane 1. A blot of the GST-GDI fraction probed with GST antibody indicates the presence of GST-GDI at 75 kDa (lane 2). A blot of GST-GDI probed with QLLQ indicates interaction between native myosin-V (196 kDa) and Rab-GDI (lane 3). (D) Motility assays in extruded squid axoplasm were used to determine the effect of Rab-GDI on actin-based vesicle transport. The number of vesicles moving per field per min (V/F/M) was measured at concentrations of 0–20 μ M GST-GDI. Vesicle transport was inhibited by 99% at 20 μ M GST-GDI.

lane 3). Therefore, these data show that myosin-V interacts with a Rab-G protein.

Motility assays were performed with GST-Rab-GDI to determine whether Rab GTPases are involved in vesicle transport in the squid giant axon. Rab-GDI blocks the exchange of GTP for GDP and thereby inactivates Rab proteins. Motile activity at 20 μ M GST-Rab-GDI decreased from 54 \pm 4 vesicles/field/min (V/F/M) in the control assay to 0.5 \pm 0.2 V/F/M (Fig. 1D). Therefore, the GST-Rab-GDI inhibited vesicle transport by 99% at this concentration. At 10 μ M GST-Rab-GDI, motile activity decreased by 86%. These data show that Rab activity is required for myosin-V-mediated vesicle transport in the axon. These data are consistent with published results showing that myosin-V is recruited to melanosomes and endosomes by Rabs 27a and 11a respectively. Our studies support the hypothesis that Rab GTPases are required for the recruitment of myosin-V to vesicles for transport.

This work was supported by NSF Grant IBN-0131470 and MBL-Shifman Award to CJD.

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