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Characterization of Myosin V Binding to Brain Vesicles*

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Myosin II and V are important for the generation and segregation of subcellular compartments. We observed that vesicular myosin II and V were associated with the protein scaffolding of a common subset of vesicles by density sedimentation, electron microscopy, and immunofluorescence. Solubilization of either myosin II or V was caused by polyphosphates with the following efficacy at 10 mM: for myosin II ATP-Mg^{2+} = ATP = AMP-PNP (5′-adenylyl imidodiphosphate) > pyrophosphate = tripolyphosphate ≫ tetrapolyphosphate = ADP > cAMP = Mg^{2+}; and for myosin V pyrophosphate = tripolyphosphate > ATP-Mg^{2+} = ATP = AMP-PNP ≫ ADP = tetrapolyphosphate > cAMP = Mg^{2+}. Consequently, we suggest solubilization was not an effect of phosphorylation, hydrolysis, or disassociation of myosin from actin filaments. Scatchard analysis of myosin V binding to stripped dense vesicles showed saturable binding with a K_m of 10 nM. Analysis of native vesicles indicates that these sites are fully occupied. Together, these data show there are over 100 myosin Vs/vesicle (100-nm radius). We propose that polyphosphate anions bind to myosin II and V and induce a conformational change that disrupts binding to a receptor.

Myosin II and V are important for the generation and segregation of subcellular compartments. The knockout of myosin II light chain kinase leads to a disruption of vesicle transport in *Drosophila* (1). Disruption of myosin V leads to accumulation of large vesicles in yeast (2–4), aggregation of melanosomes at the cell center in melanocytes (5, 6), and disruption of smooth endoplasmic reticulum in mouse cerebellular neurons (7, 8). These phenotypes suggest that myosin's role in intracellular trafficking is to bind directly to subcellular compartments and to generate force.

Many groups have proposed, based on indirect evidence, that myosin V may bind to a receptor on vesicles. In yeast and melanocytes, expression of the tail domain of myosin V leads to the same phenotypes as in the knockouts (9–11). Additionally, myosin V does not seem to be able to self-assemble to form filaments (12), but is associated with many different subcellular compartments such as the smooth ER[1] (7, 8, 13), the centrosome (14, 15), vacuoles (2, 4, 11), and synaptic vesicles (8, 16, 17). Nonetheless, because there has been no direct evidence for a protein receptor, a direct binding of myosin V to Triton X-100-insoluble lipids has also been proposed (11). In addition, although only one dimer of myosin V is required to transport beads along actin filaments (18), it is possible that many are used to transport a single cargo. Knowing if a saturable binding site is present and the number of myosin Vs that are associated with a vesicle would clarify the importance of its processive nature. Direct demonstration of saturable binding of myosin V to vesicles would elucidate the mechanism of myosin V-cargo interaction.

In preliminary studies of high density chicken brain vesicles, two proteins were solubilized by millimolar concentrations of polyphosphate anions. Microsequencing of the proteins and antibodies identified them as myosin II and V. We report that myosin V binds a saturable receptor on a detergent-resistant vesicle protein assembly. Based on the binding data and the concentration of myosin V associated with high density vesicles, we estimate that there are over 100 myosin Vs/vesicle of a radius of 100 nm.

EXPERIMENTAL PROCEDURES

Antibodies—The following primary antibodies were used in this study: a mAb against kinesin light chain (clone 9C1); a mAb against kinesin heavy chain (clone SUK4.1; Ref. 19); a polyclonal antibody against the endoplasmic reticulum protein TRAP α (a generous gift of Dr. Christopher V. Nicchitta); a mAb against chicken myosin II (clone CMII23) purchased from Developmental Studies Hybridoma Bank, an affinity-purified polyclonal antibody against myosin V (a kind gift of Dr. Richard Cheney; Ref. 20); wheat germ agglutinin-biotin (Molecular Probes); wheat germ agglutinin-FITC (molecular probes); goat anti-mouse, alkaline phosphatase-conjugated Ig (Sigma); goat anti-rabbit, alkaline phosphatase-conjugated Ig (Sigma); goat anti-rabbit Ig conjugated with Texas Red (Molecular Probes); donkey anti-mouse Ig conjugated with FITC (Molecular Probes); a mAb against kinectin (clone 160.10); avidin conjugated with alkaline phosphatase (Sigma); a mAb against γ-tubulin (Sigma); and a mAb against synaptotagmin (StressGen Biotechnologies Corp.).

Isolation of High Density Vesicles and Myosin V-enriched Supernatant—High density vesicles were collected as follows. Embryonic day 14 chick embryo brains were mixed 1:1 with 4 °C PMEE (35 mM Pipes, pH 7.4, 5 mM $MgSO_4$, 1 mM EGTA, 0.5 mM EDTA) containing 2 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, PI-H_2O (1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor), and PI-EtOH (1 μg/ml pepstatin A, 10 μg/ml TAME, 10 μg/ml TPCK), homogenized in a tight Wheaton Dounce homogenizer with 10 strokes, incubated for 10 min, and then given 10 more strokes. The homogenate was spun at 1000 × *g*, 10 min, and 4 °C in an SS34 or SA600 rotor to remove whole cells and nuclei. The loosely associated vesicle proteins were then removed with 0.1 M sodium carbonate, pH 9, followed by spinning through a 12%/36% Nycodenz step gradient for 1 h, 36,000 rpm, 225,000 × *g*, 4 °C in a SW40 rotor. The high density vesicles were collected from the 12%/36% interface. Myosin V-enriched supernatant was made by adding 10 mM pyrophosphate to the high density vesicles and spinning for 30 min at 60,000 rpm, 190,000 × *g*, 4 °C in a 100.3 rotor. For long term storage of both

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[1] The abbreviations used are: ER, endoplasmic reticulum; AMP-PNP, 5′-adenylyl imidodiphosphate; FITC, fluorescein isothiocyanate; IHP, inositol hexaphosphate; PI, protease inhibitor; Pipes, 1,4-piperazinediethanesulfonic acid; PP, disodium pyrophosphate; PPP, pentasodium tripolyphosphate; PPPP, hexammonim tetrapolyphosphate; PAGE, polyacrylamide gel electrophoresis; TAME, *p*-tosyl-L-arginine methyl ester; TPCK, tosylphenylalanine chloromethyl ketone; TRAP α, antibody against ER protein; WGA, wheat germ agglutinin; mAb, monoclonal antibody; DIC, differential interference microscopy.

the high density vesicles and the supernatants, a final concentration of 30% glycerol was then added and they were stored at −20 °C.

Density Gradient Sedimentation—To determine the nature of the interaction of myosin II and V with the high density vesicles, low speed supernatant with 0.1 M carbonate was centrifuged over a 0–36% Nycodenz gradient for 1 h at 36,000 rpm, 225,000 × *g*, 4 °C in a SW40. The high density vesicles were collected, and these were run on velocity sedimentation density gradients of 0–50% Nycodenz in the presence and in the absence of 3% Triton X-100 for 30 min, at 36,000 rpm, 225,000 × *g*, 4 °C in a SW40 rotor. Fractions from the gradient where then collected and examined by SDS-PAGE.

Electron Microscopy: Negative Staining—To observe the structure of the high density vesicles before and after Triton X-100 extraction, the vesicles from the density gradient sedimentation were adsorbed on carbon-treated EM grids for 30 s, washed with 2% uranyl acetate, and allowed to dry. The samples were observed on a transmission electron microscope (Phillips Electronic Instruments, Inc., Mahwah, NJ).

Western Blots of Density Gradient Sedimentation—To determine the epitopes associated with the high density vesicles, a low speed supernatant from homogenized brain was centrifuged over a 0–50% Nycodenz gradient. Then we ran Western blots with markers for the ER (TRAP α and kinectin clone 160.10), kinesin light chain (clone 9C1), myosin V, synaptotagmin, γ-tubulin, and the Golgi marker wheat germ agglutinin biotin (WGA-biotin). In our initial experiments, we examined each of the antibodies separately to confirm that they stained a single band by Western blot (data not shown). The exception was WGA-biotin, which labels two primary bands at about 80 and 100 kDa, and over a dozen distinct minor bands (data not shown). With this marker, the alkaline phosphatase reaction was stopped immediately after the two most prominent bands were visible.

Differential Interference Microscopy (DIC) and Immunofluorescence of High Density Vesicles—To determine the epitopes that were directly associated with the high density vesicles, they were incubated in primary antibodies and then spun through a step cushion of 12%/36% Nycodenz in PMEE with protease inhibitors, 2 mM dithiothreitol, and 1 mM ATP. The vesicles were then isolated and incubated with secondary antibodies. To remove free secondary antibody, the vesicles were spun through a second step gradient. The labeled vesicles were then viewed directly by DIC and fluorescence microscopy. Images were acquired with a STAR-1 CCD camera and saved by the program NIH Image. The images were normalized to show the same relative intensity. Photo layout was carried out in Adobe Photoshop 4.0.

For the purpose of showing statistical significance, an estimate was made of the theoretical number of vesicles that would show colocalization if the epitopes were randomly distributed on the vesicles. In each case, we counted the number of vesicles that stained for myosin V, the number of vesicles that stained for other markers, the number of vesicles that were doubly stained, and the total number of vesicles visualized by video-enhanced DIC. The percentage of vesicles that stained for myosin V was multiplied by the percentage of vesicles that stained for the other marker, which yielded the theoretical percentage of doubly stained vesicles. Student's *t* test was used to compare the theoretical and the actual number of doubly stained vesicles.

Polyphosphate Dependence of Myosin II and V Solubilization—To characterize the effect of nucleotides, polyphosphates, salts, and detergents on the solubilization of myosin II and V, high density vesicles from the 12%/36% Nycodenz interface were incubated for 20 min with 10 mM amounts of the following compounds: ATP-Mg^{2+}, ATP, Mg^{2+}, AMP-PNP, ADP, ADP + hexokinase, cAMP, inositol hexaphosphate (IHP), disodium pyrophosphate (PP), pentasodium tripolyphosphate (PPP), hexammonim tetrapolyphosphate (PPPP), 60 mM β-octylglucoside, 2% Triton X-100, 500 mM $NaCl_2$, or nothing as a control. Because ADP contains small amounts of ATP, 100 mM ADP was incubated with 1 M glucose and 2 units/ml hexokinase for 1 h and then spun through a Centricon 10 filter to remove the hexokinase. The ADP depleted of ATP was then added to a final concentration of 10 mM. The high density vesicles were then spun for 20 min at 60,000 rpm, 150,000 × *g*, in at TLA 100 rotor at 4 °C. The pellets and supernatants were examined by SDS-PAGE, and Western blots for myosin II and V. The data for the pellets exhibited a complementary pattern as compared with the supernatants and are not shown.

ATP and Pyrophosphate Concentration-dependent Solubilization of Myosin II and V—To evaluate the concentration-dependent solubilization of myosin II and V, a range of concentrations of ATP and pyrophosphate was added to high density vesicles. We then incubated them for 20 min at 4 °C, followed by centrifugation for 20 min at 60,000 rpm, 150,000 × *g*, in at TLA 100 rotor at 4 °C. The pellets and supernatants were then examined by SDS-PAGE, and Western blots for myosin II and V. Densitometry of the Western blots was used to determine the relative concentrations of myosin II and V that were in the pellets and the supernatants.

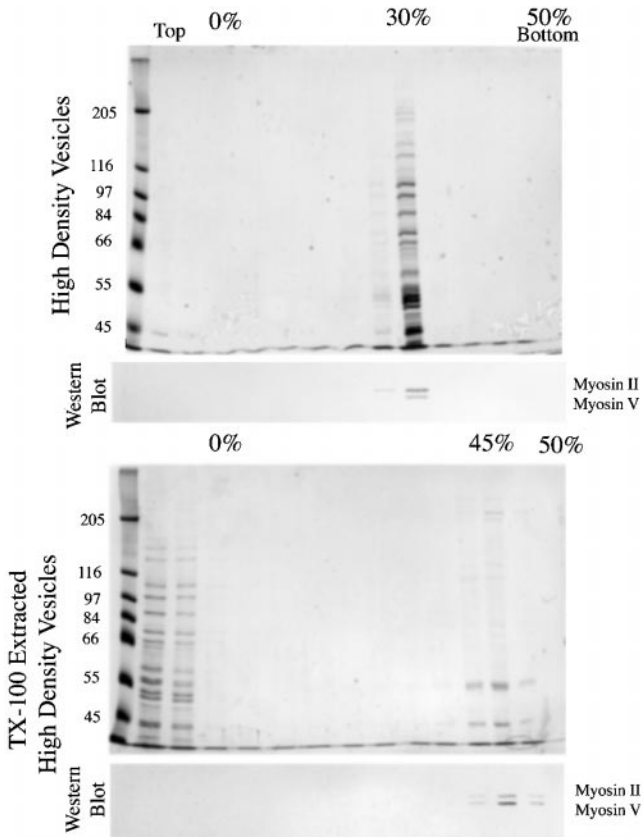

FIG. 1. **Myosin II and V associate with a Triton X-100-insoluble vesicle component.** Carbonate-washed high density vesicles were spun over a second density gradient in the presence and absence of 3% Triton X-100. Fractions from the gradient were run on a 7.5% SDS-PAGE and stained with Coomassie Blue, and by Western blot for myosin II and myosin V.

Binding of Myosin V to High Density Vesicles—Myosin V was extracted from the high density vesicles by incubation in 100 mM pyrophosphate, pH 7.0 for 30 min, followed by centrifugation through a 12%/36% Nycodenz step cushion. Enriched solutions of myosin V were centrifuged for 20 min at 30,000 rpm, 50,000 × *g*, and 4 °C in a 100.3 rotor before addition to myosin V-depleted high density vesicles. Depleted high density vesicles and different concentrations of myosin V were mixed and incubated for 1 h with rotation. The high density vesicles were then centrifuged for 20 min at 30,000 rpm, 40,000 × *g*, and 4 °C in a TLA-100 rotor. Supernatants were removed, and pellets were resuspended in an equal volume in PMEE and then solubilized in sample buffer. Protein concentrations were determined by Western blot using a known concentration of purified myosin V as a standard.

RESULTS

Myosin II and V Associate with a Large Protein Complex—Myosin V is normally associated with high density, lipid-containing vesicles (13, 16). We initially tested whether or not detergent solubilization of the lipid would solubilize the myosin V by comparing the sedimentation behavior of the high density vesicles in the presence and absence of 1% Triton X-100. In the absence of Triton X-100, the high density vesicles with myosin II and V floated as a single band. Addition of Triton X-100 solubilized over 25 proteins as identified by SDS-PAGE and moved the lipid-extracted vesicles that contained myosin II and myosin V to a higher density in the gradient (Fig. 1). These results are consistent with myosin II and V associating with a large protein complex, but not to a transmembrane protein or lipid complex.

Structure of High Density Vesicles—To determine the nature

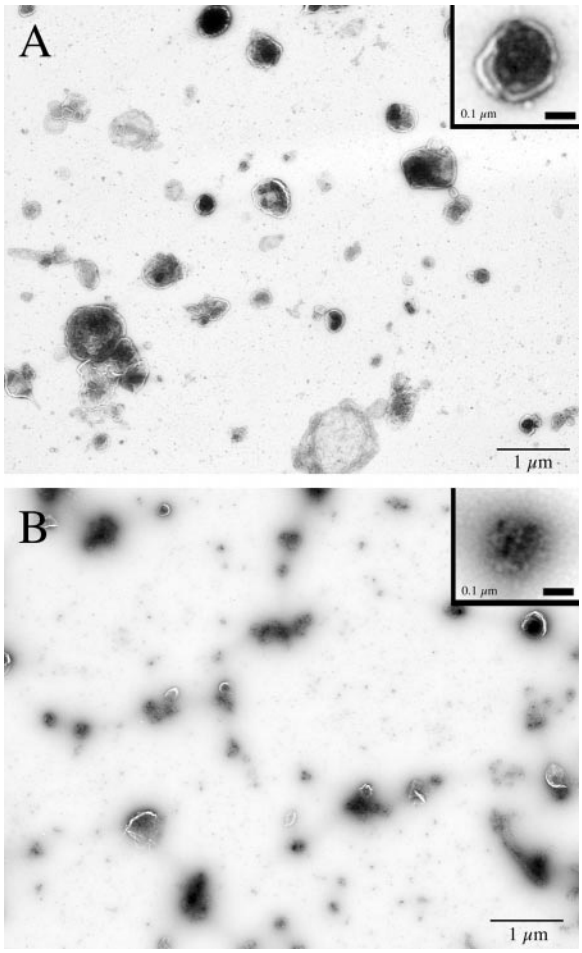

FIG. 2. **Electron microscopy of high density vesicles with and without Triton X-100 extraction.** Carbonate-washed high density vesicles in 1 mM ATP were negatively stained with 0.2% uranyl acetate and viewed by transmission electron microscopy. *Panel A* shows high density vesicles, and *panel B* shows high density vesicles that have been extracted with 2% Triton X-100.

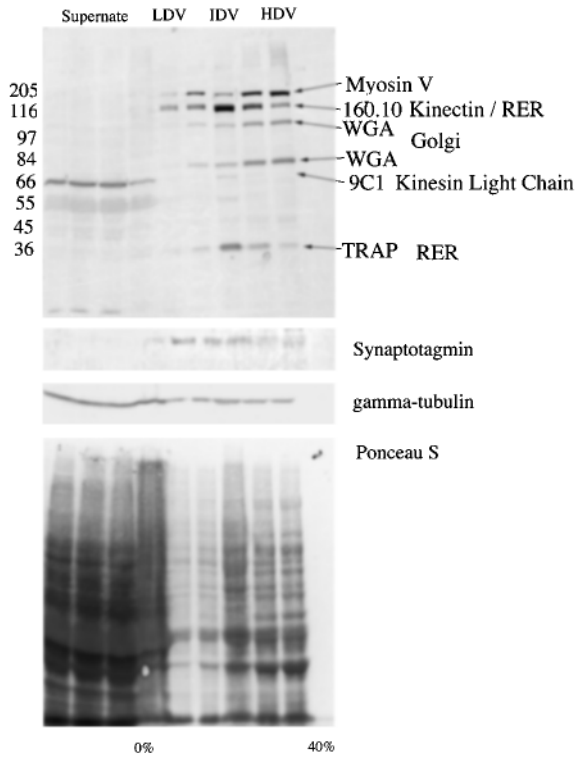

FIG. 3. **Myosin V is enriched in the high density vesicle fraction.** Crude cytosol was fractionated on a linear 0–40% Nycodenz density gradient. Fractions were transferred to nitrocellulose and probed with the markers as indicated. Myosin V was predominantly enriched with the Golgi marker (WGA) in the high density vesicles, and there was a small but consistent fraction found between the low density vesicle and the intermediate density vesicle. Kinesin was associated with intermediate density vesicles that were enriched for the ER proteins kinectin and TRAP α. Synaptotagmin was enriched in the low density vesicles, and γ-tubulin was distributed through the gradient. In each case, antibodies were run on separate blots to verify specificity.

of the complex remaining after Triton X-100 extraction, the high density vesicles were negatively stained with 2% uranyl acetate and examined using transmission electron microscopy. High density vesicles in the absence of Triton X-100 varied between 70 and 300 nm in diameter and had a distinct bilayer structure (Fig. 2*A*). Addition of 2% Triton X-100 followed by centrifugation through a step gradient left complexes of protein with about the same size and shape as the original high density vesicles. In some cases, these protein complexes were associated with partially extracted membranes (Fig. 2*B*). Nonetheless, microtubules, actin filaments, or other filamentous structures were absent in both the control and Triton X-100-extracted vesicles (Fig. 2, *A* and *B*). This is consistent with the results from the sedimentation experiment and with previous work that has shown that myosin V interacts with small dense vesicles (10, 13).

Myosin II and V Are Not Associated with Lipid Rafts—Catlett and Weisman (11) have proposed that myosin V could interact with the high density vesicles via Triton X-100-insoluble lipid rafts. To test this, the high density vesicles were incubated with 60 mM β-octylglucoside, which has been demonstrated to solubilize lipid raft associated proteins (21–25). The high density vesicles were then centrifuged at 60,000 rpm, 150,000 × *g*, 20 min, and 4 °C in a TLA-100 rotor. Triton X-100-extracted vesicles were run as a control (Fig. 6, *lanes L* and *M*). The supernatant was then examined by Western blot to determine the solubilization of the myosin. Neither of the detergents solubilized over 5% of either myosin II or V. The lack of solubilization by Triton X-100 is consistent with the results of several groups (11, 16, 26). The lack of solubilization with β-octylglucoside has not been previously reported, and this supports the conclusion that myosin II and V are not associating with Triton X-100-insoluble lipid rafts, and must instead be interacting with protein complexes such as those observed by electron microscopy above.

On Density Gradients, the Golgi and Myosin V Are Enriched in the High Density Vesicles—To aid in identifying the subcellular compartment that the myosin V-containing vesicles were derived from, we stained for proteins associated with the high density vesicles. After separation of low speed homogenates of chick brain on a density gradient, we examined them with Western blots (Fig. 3). We found that over 66% of the total myosin V was enriched in the fractions containing high density vesicles. Additionally, a smaller amount (~16%) was in a fraction between the low and intermediate density vesicles. We next tested to see which components were tightly associated with the myosin V vesicles.

Prekeris and Terrian (17) have reported an association of myosin V with synaptic vesicles. Therefore, it was of interest to determine if a marker for synaptic vesicles showed enrichment in the same fractions that were enriched in myosin V (Fig. 3). We found that the synaptic marker synaptotagmin was enriched in the low density vesicles, which contain a small fraction of the myosin V. Nonetheless, there was little synaptotagmin with the myosin V in the high density vesicles. It is

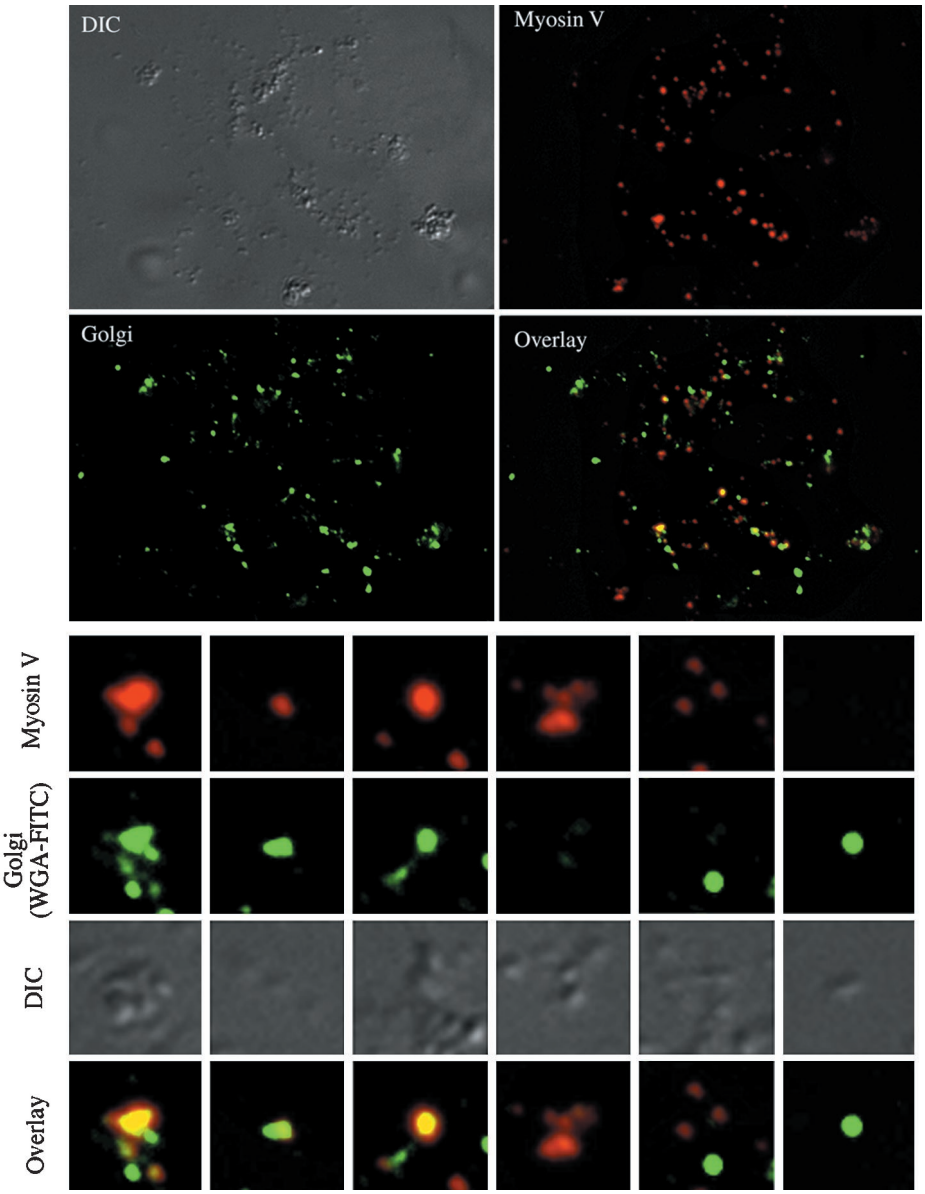

FIG. 4. **Myosin V and the Golgi are only found associated as aggregates on high density vesicles.** High density vesicles were incubated with primary antibody and then spun through a Nycodenz cushion. Secondary antibody and WGA-FITC were then added, and the vesicles were spun through a second cushion. Vesicles were viewed directly (magnification, ×100) with an Axiophot. The Golgi was stained *green* with WGA-FITC, and myosin V was stained *red* with goat anti-rabbit conjugated with Texas Red.

possible that the myosin V that is in the low density fractions is associating with the synaptobrevin-synaptophysin complex. Nonetheless, the myosin V that we have focused on in our studies does not seem to be associated with synaptic vesicles.

In addition, myosin V has been reported to be associated with neuronal specific kinesin as assayed by the yeast two-hybrid system (27). Therefore, we determined the localization of kinesin relative to myosin V. We found that over 95% of the kinesin was in the soluble fraction, and the remainder (3%) was associated with the intermediate density vesicles (Fig. 3). These vesicles were also enriched with the ER markers kinectin and TRAP α. Again, the majority of the myosin V is associated with the high density vesicles, and therefore, it does not appear that kinesin and myosin V are enriched in the same population of vesicles *in vivo*.

Myosin V has been shown to interact with centrosomes (14, 15). We were curious if the high density vesicles were enriched in a marker for the centrosomes. Therefore, we examined the distribution of γ-tubulin (28, 29). We found that γ-tubulin was distributed throughout the gradient, and did not show enrichment in the high density vesicles (Fig. 3). Therefore, we suggest that myosin V is not associating with the high density vesicles via an interaction with centrosomes.

Myosin II has been reported to be indirectly associated with the Golgi (30, 31). Therefore, we wanted to determine if myosin V might show an association with the Golgi. We chose to assay the distribution of the Golgi using WGA-biotin. We found that this marker is predominantly enriched in the high density vesicles, and suggests the myosin V may be associated with Golgi (Fig. 3).

Myosin V Does Not Colocalize with Golgi but Does Colocalize with Myosin II—From the gradients, it appeared that myosin V may be associated with the Golgi. To test for direct association, we co-stained high density vesicles with both an anti-myosin V antibody (rhodamine secondary) and WGA-FITC (Fig. 4). Myosin V stained 25 ± 9% (average ± standard deviation) and WGA-FITC stained 25 ± 9% of the total number of counted vesicles. Of the vesicles that stained for myosin V, 18 ± 13% costained with WGA-FITC, where 25 ± 9% would have been expected to be double labeled by chance. Visual examination of the vesicles revealed that colocalization predominately occurred where large vesicles were seen by DIC, and the immunofluorescent staining of the two epitopes did not directly overlie each other. Therefore, we conclude that vesicles that contain myosin V or the Golgi may associate in aggregates. In addition, when we compared the staining of ER markers with that of

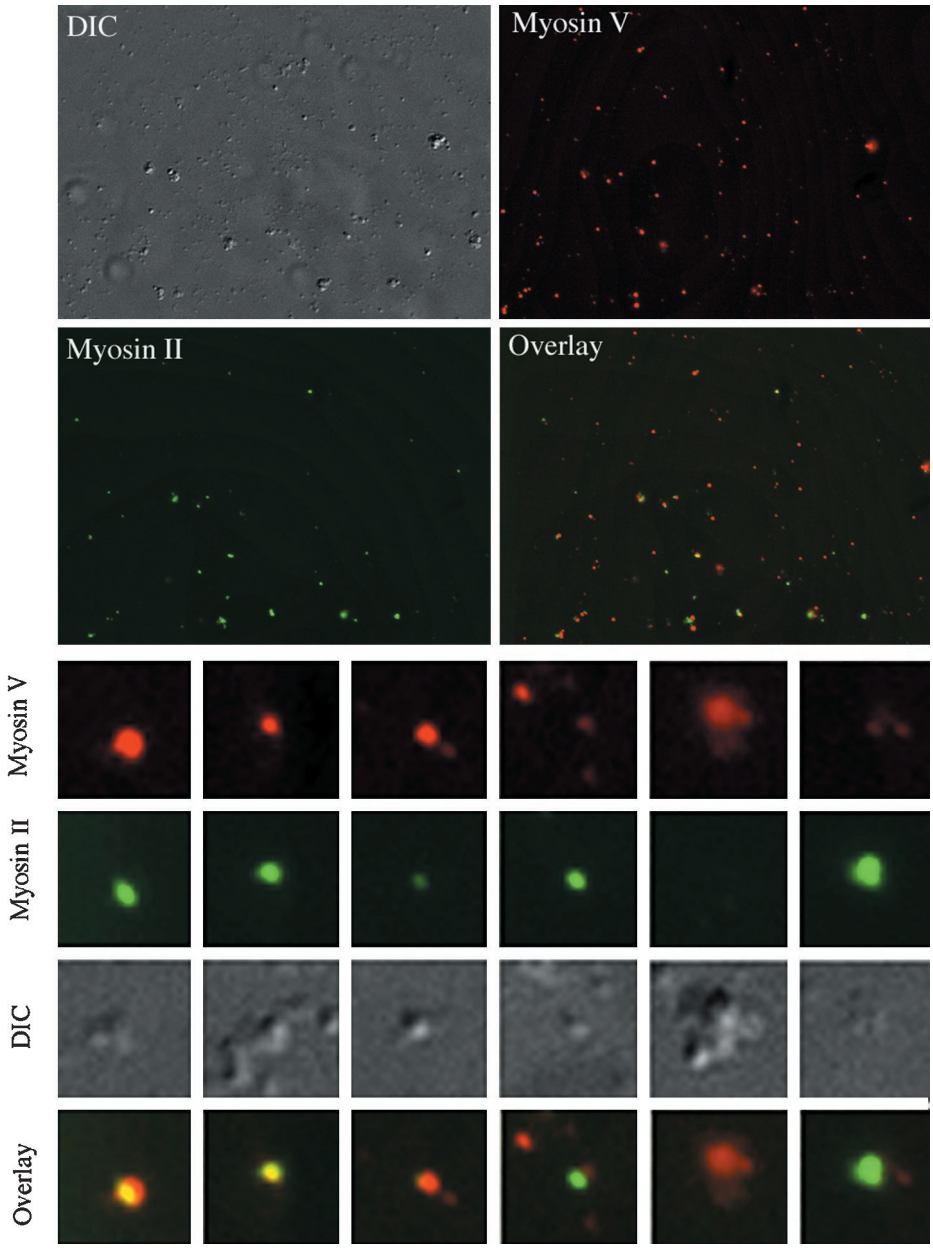

FIG. 5. **Myosin II and V colocalize.** High density vesicles were incubated with primary antibody and then spun through a Nycodenz cushion. Secondary antibodies were then added, and the vesicles were spun through a second cushion. Vesicles were viewed directly (magnification, ×100) with an Axiophot. Myosin II was stained *green* with goat anti-rabbit conjugated with FITC, and myosin V was stained *red* with goat anti-rabbit conjugated with Texas Red.

myosin V, we also did not find overlap, as would be expected from the gradients shown in Fig. 3 (data not shown).

In contrast, myosin II and V showed significant colocalization. Myosin V stained 25 ± 9% (average ± standard deviation) and myosin II stained 5 ± 2% of the total number of counted vesicles. Of the vesicles that stained for myosin V, 21 ± 8% costained with myosin II, but nearly all of the myosin II vesicles stained for myosin V (Fig. 5). Statistically, there was a significant positive correlation of staining ($p > 0.03$; $n = 7$). In addition, visual examination of the immunofluorescent staining of myosin II and V showed that they clearly overlapped. This is consistent with myosin II and V associating on the same vesicles.

Myosin II and V Are Selectively Solubilized by Di- and Triphosphates, but Not cAMP, Tetrapolyphosphate, or 500 mM NaCl—We were interested in the nature of association between the high density vesicles and the myosin II and V. Therefore, we isolated carbonate-washed high density vesicles and added the following compounds all at a pH of 7.2 and at a concentration of 10 mM for solubilization: ATP-Mg^{2+}, ATP, Mg^{2+}, AMP-PNP, ADP, ADP + hexokinase (to determine if the release required phosphorylation or hydrolysis), cAMP (to determine if the release was sensitive to the adenosine backbone), IHP (to determine what moiety of the ATP was important for release), sodium pyrophosphate, pentasodium tripolyphosphate, and hexammonim tetrapolyphosphate (to determine if di- and triphosphates alone were capable of releasing the myosin II and V) (Fig. 6, *lanes B–L*). Myosin II and V in the supernatants and pellets were then examined by SDS-PAGE and Western blot. The ability of the compounds to solubilize myosin II, from best to worst, was: (ATP-Mg^{2+} = ATP = AMP-PNP > IHP > PP = PPP ≫ PPPP = ADP + hexokinase > cAMP = Mg^{2+}). With respect to myosin V, the order of solubilization was (PP = PPP > ATP-Mg^{2+} = ATP = AMP-PNP > IHP ≫ ADP + hexokinase = PPPP > cAMP = Mg^{2+}). The ADP was treated with hexokinase to remove residual ATP; nonetheless, the small amount of release by ADP + hexokinase maybe due to the presence of trace amounts of ATP.

The patterns of solubilization of the myosin II and V are consistent with the hypothesis that di- and triphosphates bind to specific sites on myosin II and V and change their conformation, but not with the hypothesis that the solubilization is due

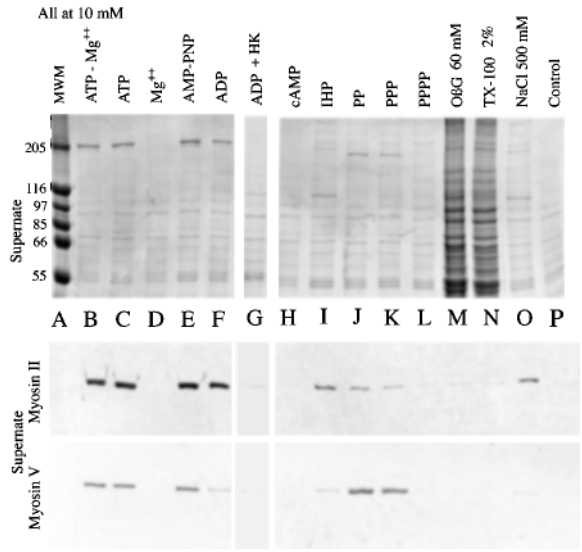

FIG. 6. **Myosin II and V solubilization by nucleotides and detergents.** High density vesicles were isolated, and the compounds shown were added. The vesicles were incubated for 20 min, then spun at 20 min at 60,000 rpm, 150,000 × *g*, in a TLA 100 rotor at 4 °C. Supernatants were collected and examined by SDS-PAGE and by Western blot with antibodies against myosin II and V.

to hydrolysis, phosphorylation, or disruption of an actin-myosin head interaction. In addition, the pattern of solubilization is not consistent with a nonspecific ionic disruption. Tetrapolyphosphate and inositol hexaphosphate, which have the greatest charge of the compounds tested, were relatively ineffective in solubilizing the myosin II and V. Likewise, 500 mM NaCl did not solubilize any of the myosin V and only a modest amount of the myosin II (Fig. 6).

The interaction of myosin V with synaptophysin and synaptobrevin is selectively disrupted by Mg^{2+} (17). Even though we did not find the synaptic vesicle marker synaptotagmin in the high density vesicles, we wanted to test if Mg^{2+} could solubilize myosin V from them. In the assay described above, we examined the effect of adding 10 mM ATP-Mg^{2+}, 10 mM ATP, or 10 mM Mg^{2+} on the solubilization of myosin V by SDS-PAGE and Western blot (Fig. 6, *lanes B*, *C*, and *D*). We found that ATP alone was sufficient to solubilize myosin V from the high density vesicles, and that 10 mM Mg^{2+} had no effect either by itself or in combination with ATP, ruling out a Mg^{2+}-dependent binding to the vesicles.

ATP and Pyrophosphate-dependent Solubilization of Myosin II and V—Complete solubilization of myosin II or V over a narrow concentration range of ATP or pyrophosphate would be consistent with binding to a single receptor. We characterized the concentration-dependent solubilization of myosin II and V by densitometry of Western blots (Fig. 7). In the absence of ATP, 99% of the myosin II and V pelleted with the high density vesicles. At the maximum ATP concentration of 100 mM, 65% of the myosin II and 95% of the myosin V could be solubilized. The concentration of ATP that solubilized half of the myosin II was 0.08 mM, and for the myosin V it was 3 mM. Similarly, at the maximum pyrophosphate concentration, 90% of the myosin V and 80% of the myosin II could be solubilized. The concentration of pyrophosphate that solubilized half of the myosin V was 2 mM, and for myosin II it was 60 mM. For myosin II, these data are consistent with the presence of two different binding sites, one site that is sensitive to nucleotides and polyphosphates and a second site that is not. In contrast for myosin V, these data are consistent with it binding to a single receptor through a nucleotide- or polyphosphate-sensitive mechanism.

Myosin V Binds to Vesicles in a Reversible and Saturable Manner—We wanted to determine if myosin V was binding to a saturable receptor on the high density vesicles. In these experiments, we mixed myosin V at concentrations ranging from 1.5 to 150 nM with 0.5 mg/ml high density vesicles that had been depleted of myosin V using 100 mM pyrophosphate. After incubation, the high density vesicles were centrifuged and the concentration of myosin V in the supernatants and pellets was determined (Fig. 8). As a control, myosin V without high density vesicles was centrifuged in parallel. In the absence of high density vesicles, myosin V did not pellet, and therefore was not undergoing self-assembly. As the concentration of myosin V was increased, the amount of myosin V that pelleted with the high density vesicles increased initially and then leveled off, while the amount of free myosin V continued to increase. This is evidence of binding to a saturable receptor.

To determine the affinity of the myosin V-vesicle interaction and the number of binding sites per vesicle, a Scatchard analysis was performed. In the analysis, free myosin V was undetectable below 30 nM; therefore, those values were excluded in the determination of the apparent affinity (K_m). The K_m of myosin V for the vesicles was 10 nM, and at saturation there was 80 nM myosin V bound/1 g/liter of high density vesicles.

Estimation of the Number of Myosin Vs Bound per Vesicle—The number of myosin receptors per vesicle was estimated by dividing the concentration of myosin receptors by the concentration of vesicles. The concentration of myosin dimer receptors was determined from the Scatchard analysis (Fig. 8) and was 40 nM for a 1 g/liter solution of high density vesicles. The concentration of vesicles (V_{conc}) was calculated using the equation below.

$$V_{conc} = C_{prot}/(4/3)\pi r^3 N\rho F \qquad \text{(Eq. 1)}$$

The protein concentration (C_{prot}) was 1 g/liter. The median vesicle radius (r) was estimated to be 100 nm/vesicle from our electron micrographs (Fig. 2). N is Avogadro's number (6.02×10^{23} vesicles/mol). The vesicle density (ρ) was 1.3 g/ml as estimated from our density gradients (Fig. 1). The fraction of vesicle mass that was protein (F) was estimated to be 0.21. This is based on the assumption that 70% of the vesicle mass is H_2O (32), and the remaining dry mass is a combination of proteins and lipids. Because it is known that protein density is 1.4 and lipid density is 1.05, based on the vesicle density of 1.3, we can estimate algebraically that the percentage of protein in the dry mass of the vesicles is 25/35 × 100% = 71%, and therefore the mass of the protein in the hydrated vesicles is 0.21 of the total.

Based on these calculations, the vesicle concentration is 1.5 nM and the receptor concentration is 40 nM, which yields an unadjusted 27 myosin dimer receptors/100 nm in radius vesicle on average. Because only 25% of the vesicles stained for myosin V (Fig. 4), the number of myosin V dimer receptors per vesicle would be 109. The estimation of the number of receptors per vesicle is very sensitive to the radius of the vesicles, and it is logical that smaller vesicles would also have few myosin V receptors. For comparison, the number of receptors that would be associated with a vesicle 50 nm in radius is 13, and for a 30 nm in radius vesicle there would be only 3 myosin V receptors.

The level of receptor saturation *in vivo* was estimated by dividing the myosin V concentration by the myosin V receptor concentration. The amount of myosin associated with the vesicles was determined by densitometry and was approximately 1.5% of the total protein (Fig. 1). For a 1 g/liter solution of high density vesicles, this translates to a myosin V (mass = 400,000 kDa) concentration of 37.5 nM. The receptor concentration from the Scatchard analysis was 40 nM; therefore, regardless of the size of the vesicles, the receptors are approximately 94% saturated *in vivo*.

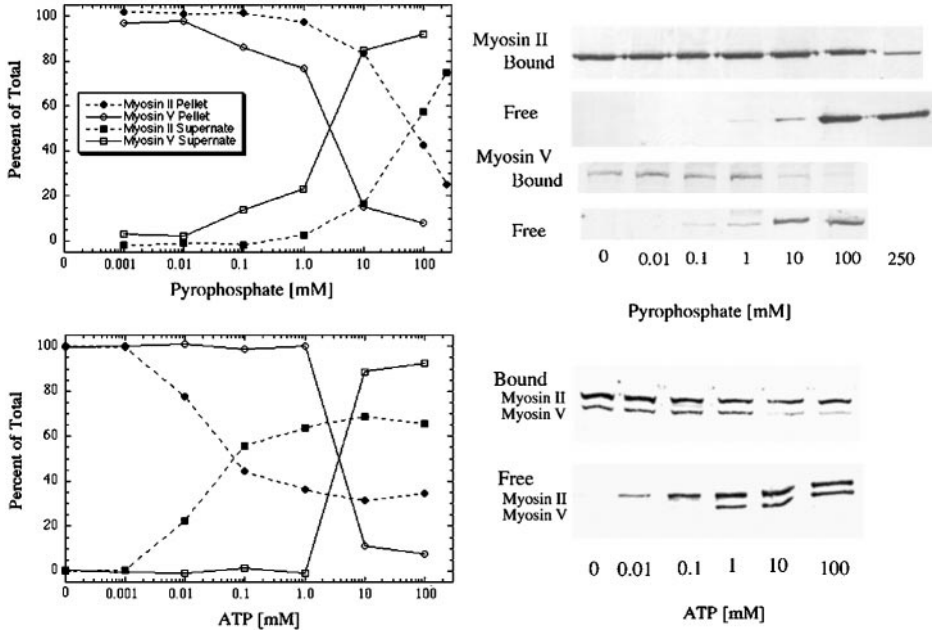

FIG. 7. **Myosin II and V concentration-dependent solubilization by pyrophosphate and ATP.** Carbonate-washed high density vesicles were incubated with the specified concentrations of pyrophosphate and ATP, and the relative amounts of myosin II and V in the supernatant and pellets were calculated. The graph shows the normalized values.

DISCUSSION

The results of these experiments indicate that myosin V reversibly binds to a high affinity saturable receptor on a subpopulation of high density vesicles. Myosin V binding and solubilization, like myosin II filament assembly, is dependent upon a polyphosphate anion binding site(s). These results and the morphology of the vesicles before and after detergent extraction are inconsistent with the hypotheses that myosin V self-assembles to form mini-filaments or that myosin V reversibly interacts with vesicular actin through its head domain. Rather, it seems that myosin V and perhaps myosin II bind to a multimeric complex of proteins on the vesicle surface.

Myosin V binding to vesicles has been documented in several other systems (13, 16). The methods used to isolate the vesicles in those cases and the vesicles themselves were similar to the ones that we have characterized. Nonetheless, we were concerned that an actin-myosin or a myosin filament assembly could be responsible for the binding. It is well established ATP weakens the binding of S1 muscle myosin to actin filaments by 3000-fold, where AMP-PNP and pyrophosphate weaken this interaction by 300-fold (33). We found that pyrophosphate and AMP-PNP weaken the interaction between the vesicles and myosin II and V at equal or greater levels than ATP. In addition, the lack of obvious actin filaments in the electron micrographs suggests that the association of the myosin with the vesicles is not through the head domain binding to actin. The failure of myosin V to pellet in the absence of high density vesicles is inconsistent with filament formation. What is left is the possibility that myosin II and V bind large lipid-associated protein complexes.

After detergent extraction, myosin II and V sedimented rapidly to a high density, indicating that they were associating with a multisubunit protein complex (Fig. 1). Consistent with this hypothesis, at the electron microscope level, Triton X-100 removes the lipid bilayer, but complexes of the size and shape of the original vesicles remain (Fig. 2). One explanation for these results is that myosin II and V could be associating with lipids that are resistant to extraction by Triton X-100 (11, 22). To address this, we examined the behavior of myosin II and V in the presence of β-octylglucoside and found that they were both insoluble (Fig. 6, *lane L*). In summary, the Triton X-100 and β-octylglucoside extraction studies rule out the possibility that myosin II or V directly interacts with lipids, lipid bound receptors, or lipid rafts. Together, these data support the conclusion that myosin II and V bind to a multisubunit protein complex that is associated with lipids such as coated vesicles.

Previous observations of myosin V containing vesicles by immunogold electron microscopy showed that two general size classes of vesicles had myosin V bound to their surface: large vesicles (500–800 nm) and small vesicles <200 nm (10, 13, 16), and our observations are similar (Fig. 2). In agreement with several other reports, we found that the vesicles which stained for myosin V did not exclusively contain markers for endoplasmic reticulum (10, 13), synaptic vesicles (17), or centrosomes (14, 15) (Fig. 3). While these results are negative, they are consistent with the theory that myosin V does not associate with a single subcellular compartment, but instead may be involved with inter-compartmental trafficking.

The nature of the interaction between myosin II and V and the protein complex coating the vesicle appeared to be similar to myosin II filament assembly. Myosin filaments are solubilized at millimolar concentrations of pyrophosphate, AMP-PNP, and ATP (34). This was not a general effect of ionic strength, since under the same conditions 500 mM NaCl does not significantly solubilize myosin V (Fig. 6). Furthermore, solubilization was not a result of a nonspecific charge effect of the polyphosphates because tetrapolyphosphate does not solubilize myosin II and V nearly as well as the di- and triphosphates. Differential solubilization of myosin II and V was also observed with respect to the concentrations of ATP or pyrophosphate needed for 50% solubilization. The selective nature of the different anions indicated that the polyanion binding sites were different between myosin II and V. In addition, over 95% of myosin V was capable of solubilization, and this occurred over a narrow concentration range, whereas only 60% of the myosin II was solubilized by ATP. For myosin II, these data suggest it is bound to two sites, one polyphosphate-sensitive and the other insensitive. In contrast, myosin V appears to bind to a single receptor through a nucleotide- or polyphosphate-sensitive mechanism. We suggest that both myosin II and V binding to the vesicle coat are regulated through a tail assembly mechanism similar to myosin II filament assembly.

Myosin V appears to be binding to a single type of receptor. We found clear evidence of a high affinity (10 nM) saturable myosin V binding site, with 80 nM receptor/1 g/liter of vesicles. For a vesicle with a radius of 100 nm, we estimate that there

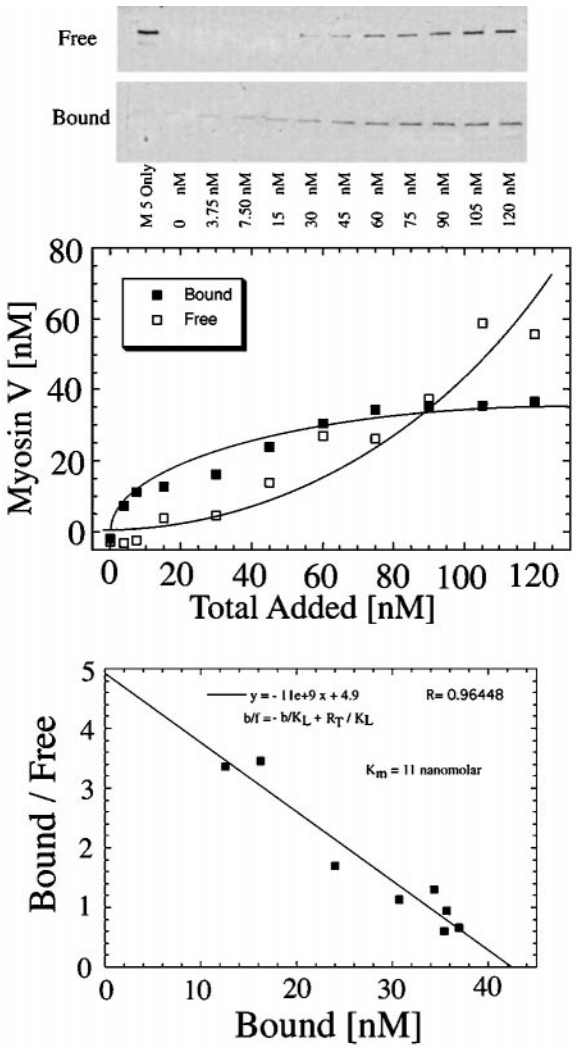

FIG. 8. **Myosin V binds to a high affinity saturable receptor.** Carbonate-washed high density vesicles (100 μg) were extracted of myosin V and then incubated with a range of myosin V concentrations. The high density vesicles were pelleted, and the concentrations of myosin V in supernatants and pellets were determined by Western blot. Binding data were examined by Scatchard analysis. The affinity of interaction is K_m = 10 nM with over 100 sites/100 nm in radius vesicle.

are approximately 109 myosin V receptors/vesicle and that they at fully occupied *in vivo*. To interpret these data, we need to understand more about the function of this fraction of the myosin V *in vivo*. Several scenarios seem likely with regard to myosin V; 1) this could be an inactive storage pool of motor, 2) myosin V could aid in shaping and forming vesicles by facilitating coat assembly or anchoring the complex, or 3) this could represent the transport complex. We believe that the first possibility is unlikely because such an abundant and regular structure is likely to have other functions.

Recent studies have highlighted myosin V's role as a linker between the actin cytoskeleton and subcellular organelles. In melanocytes, a lack of myosin V or expression of the tail results in a phenotype where the melanosomes are concentrated in the center of the cell. Video microscopy of the melanosomes has revealed that they are transported up and down microtubules. The disruption of myosin V does not decrease their motility, but instead prevents the melanosomes from interacting with the actin cytoskeleton at the edge of the cell (9). The importance of having several hundred myosin Vs associated with a single cargo might be to ensure that organelles could generate enough force to tear them from the microtubule cytoskeleton. Additionally, myosin V may be part of the coat complex and may have a role in stabilizing or building the coat structure.

If myosin V on these vesicles is an active motor, the high number of molecules per vesicle is surprising, since myosin V has been shown to be a processive motor (18). In cases where a small organelle requires transport through a meshwork of actin, myosin V is a natural candidate and many motors may be necessary to break through the actin mesh. Alternatively, the presence of many molecules belies a cooperative process wherein activation could be dependent upon small changes in conditions. In the case of smaller synaptic vesicles (30 nm radius), only a couple of myosin molecules could be bound and processivity would be important. Further study is required to establish whether myosin V is predominately involved in motility, stabilization, or some other function.

We propose that the mechanism of regulation of myosin II and V binding to vesicles is analogous to the mechanism of myosin II filament assembly. α-Helical coiled-coils are important for the assembly of many different protein complexes, and a similar mechanism could be adapted for vesicle surfaces. Because self-assembly plays such an important role in the polymerization of myosin filaments, we speculate that the receptors for myosins may have significant homology to the tail of myosins. Co-opting myosin II's mechanism for the regulation of filament formation seems a reasonable way to regulate the binding of myosin II and V to receptors. Much more information is needed to understand the function of this major pool of the myosin V.

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