Myosin V Plays an Essential Role in the Thyroid Hormone-dependent Endocytosis of Type II Iodothyronine 5’-Deiodinase*

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In astrocytes, thyroxine modulates type II iodothyronine 5’-deiodinase levels by initiating the binding of the endosomes containing the enzyme to microfilaments, followed by actin-based endocytosis. Myosin V is a molecular motor thought to participate in vesicle trafficking in the brain. In this report, we developed an in vitro actin-binding assay to characterize the thyroid hormone-dependent binding of endocytotic vesicles to microfilaments. Thyroxine and reverse triiodothyronine (EC50 levels ~1 mM) were >100-fold more potent than 3,5,3’-triiodothyronine in initiating vesicle binding to actin fibers in vitro. Thyroxine-dependent vesicle binding was calcium-, magnesium-, and ATP-dependent, suggesting the participation of one or more myosin motors, presumably myosin V. Addition of the myosin V globular tail, lacking the actin-binding head, specifically blocked thyroid hormone-dependent vesicle binding, and direct binding of the myosin V tail to enzyme-containing endosomes was thyroxine-dependent. Progressive NH2-terminal deletion of the myosin V tail and domain-specific antibody inhibition studies revealed that the thyroxine-dependent vesicle-tethering domain was localized to the last 21 amino acids of the COOH terminus. These data show that myosin V is responsible for thyroid hormone-dependent binding of primary endosomes to the microfilaments and suggest that this motor mediates the actin-based endocytosis of the type II iodothyronine deiodinase.

Type II iodothyronine deiodinase (D2)1 is a membrane-bound enzyme that catalyzes the generation of T3 from T4, and is the major source of T3 in brain, pituitary, and brown adipose tissue (1, 2). In the brain, D2 levels are dynamically regulated by thyroid hormone (3–6). The thyroid hormone-dependent regulation of D2 observed in vivo is mimicked in cAMP-stimulated astrocytes in culture (4). In the absence of thyroid hormone, D2 levels are elevated, enzyme turnover is slow, and the microfilaments are disrupted (4, 7). Addition of T4 or rT3, but not the transcriptionally active T3, causes the rapid restoration of the microfilaments and activates actin-based endocytosis of D2-containing vesicles, leading to a rapid fall in D2 levels in the cell activity (5, 8, 9). Importantly, repolymerization of the microfilaments in the absence of thyroid hormone does not alter D2 turnover or activate the actin-based endocytosis of D2-containing vesicles (5, 8, 9), suggesting that thyroid hormone independently regulates actin-based endocytosis. Inhibitor studies showed that disruption of the actin cytoskeleton by dihydrocytochalasin or depletion of cellular ATP stores block the T4-induced loss of D2 (4, 5) indicating that intact microfilaments and an energy source are required for the dynamic regulation of the turnover of this membrane-bound enzyme.

One of the first steps in the T4-dependent regulation of astrocyte D2 activity is the hormone-induced binding of D2-containing vesicles to filamentous actin (F-actin). This is rapidly followed by the translocation of the actin-bound vesicle to the perinuclear space (5, 10). Myosin motor proteins mediate the translocation of vesicle on actin fibers. Myosins comprise a superfamily of actin-binding, Mg2+-ATPase, motor proteins (11), and unconventional myosins are found in virtually all cell types, where they participate in cell contraction, cell motility, and vesicle trafficking (12). Four unconventional myosin family members (I, V, VI, and VII) have been reported to participate in membrane trafficking (12, 13). Myosin V is the most abundant of this subset in brain, where it is found to be associated with vesicles in nerve terminals (13, 14). Myosin V forms calcium-dependent complexes with the synaptic vesicle proteins, synaptobrevin and synaptophysin (14). Although this motor protein does not appear to associate with the mature synaptic vesicle, ultrastructure analysis showed that larger-sized, SV2-positive vesicles in the synapse were decorated with myosin V (15), suggesting that myosin V is bound to endosomes and/or to recycling synaptic vesicles (16).

In this study, we examined the participation of myosin V in the thyroid hormone-dependent binding of D2-containing vesicles to the actin cytoskeleton. Using both the native, affinity-radiolabeled D2, and exogenous expression of a GFP-tagged D2 fusion protein, we show that an actin-bound protein with calcium-dependent ATPase activity tethers the D2-containing endosome to F-actin. Deletion analysis and antibody inhibition studies showed that the COOH-terminal 21 amino acids anchored the D2 vesicle to the myosin motor in a hormone-dependent manner. These data suggest that the processive myosin V motor participates in the T4-dependent actin-based endocytosis of D2.
**Experimental Procedures**

**Materials**—T₄, Triton X-100, ATP, Bt₂cAMP, hydrocortisone, colchicine, bovine serum albumin, and rabbit anti-actin IgG were obtained from Sigma (St. Louis, MO). DMEM, antibiotics, Hank’s solution, and trypsin were purchased from Life Technologies, Inc. (Gaithersburg, MD). Acrylamide was purchased from National Diagnostics (Atlanta, GA). TEMED and ammonium persulfate were purchased from Bio-Rad (Richmond, CA). Hybond ECL nitrocellulose was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL); horseradish peroxidase (HRP)-conjugated goat, anti-rabbit IgG was obtained from Promega (Madison, WI); rabbit anti-GFP IgG was from CLONTECH (Palo Alto, CA). The Lumiglo chemiluminescent detection system was obtained from Kirkegaard and Perry (Gaithersburg, MD). BrAc[125I]T₄ was synthesized as described previously (17). The TNT-coupled transcription-translation kit was purchased from Promega (Madison, WI). Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, MA).

**Culture Conditions**—Astrocytes were prepared from 1-day-old neonatal rats as described previously (18) and grown in growth medium composed of DMEM supplemented with 10% supplemented bovine calf serum, 50 units/ml penicillin, and 90 units/ml streptomycin. Cells were grown to confluence in 75-cm² culture flasks in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C and used at passages 1–3.

**Actin Binding Assay**—Cell lysates with intact microfilaments (F-lysate) were prepared from thyroid hormone-deficient astrocytes treated for 24 h with 10 μM retinoic acid in serum free media as detailed previously (10). Cell lysates with BrAc[125I]T₄-labeled p29 vesicles (V-lysate) were prepared from cAMP-stimulated astrocytes in serum free medium with 2 mM BrAc[125I]T₄ as described above (5). Microtubules were depolymerized in all cells using 10 μM colchicine for 30 min before cell isolation. Cells were then scraped from the flask, collected by centrifugation (500 × g for 5 min), washed with phosphate-buffered saline (pH 7.4), and the cell pellets were lysed by two freeze-thaw cycles (5, 8). Lysates could be stored at −70 °C for up to 4 weeks without loss of hormone-dependent actin binding. F- and V-lysates (100 μg of protein each) were combined on ice, 100 mM T₄, 10 mM T₃, or 10 mM T₄, were added, and the mixtures were incubated for 20 min at 37 °C. Mixtures were then chilled on ice for 2 min, Triton X-100 (0.5% v/v, final concentration) was added, and the soluble (Triton supernatant) and particulate (Triton pellet) fractions were separated by centrifugation at 4 °C for 65,000 × g min. The distribution of [125I]-labeled p29 between the Triton supernatant and Triton pellet was determined by SDS-PAGE analysis.

**Antibody Preparation**—Synthetic peptides corresponding to the last 22 amino acids COOH-terminal to myosin V (NH₂-YSLEATLITQIPSGLGGFIARY-COOH) were synthesized by the Peptide Synthesis Core at the University of Massachusetts Medical School. An NH₂-terminal tyrosine residue was added to facilitate dianimobenzidine coupling to KLH and for radiiodination. The peptide-KLH conjugate (750 μg of KLH conjugated peptide) was mixed with an equal volume of Freund’s adjuvant and injected intradermally at 20 sites on the back of 2.2-kg female New Zealand white rabbits. Antibodies were also raised against an internal myosin V domain corresponding to the last IQR domain and the coiled-coil region (residues 892 to 1040, myosin Vmid). Polymerase chain reaction amplified myosin V cDNA was prepared using site-specific, 20-mer oligonucleotides and the −500-bp fragment was cloned into the EcoRV site of the pThioHis B prokaryotic expression vector (Invitrogen, San Diego, CA). The fusion protein was synthesized in isopropyl-1-thio-β-D-galactopyranoside-induced Escherichia coli. The myosin Vmid fusion protein was purified by Ni-Sepharose from cell lysates according to the manufacturer’s instructions. Approximately 75 μg of myosin Vmid was diluted 50:50 with complete Freund’s adjuvant used to immunize rabbits as described above.

The specificity of the two rabbit anti-myosin V antisera was documented by immunoblot analysis. Brain homogenates were prepared from normal, heterozygous (myosin V mid/) and myosin V-deficient, homozygous dilute mouse (myosin V mid/–). Both antibodies recognized a 190-kDa protein in the brain homogenates containing myosin V (heterozygotes) but showed no immunoreactive band in the homogenates of dilute mice, which lacks myosin V.

**Immunoblotting**—Total cell protein was measured by the Bradford dye binding assay (Sigma), and 20–50 μg of cellular protein was reduced, denatured, and separated by SDS-PAGE according to the method of Laemmli (19). Resolved proteins were transferred to Hybond membranes by electrotransfer using a semidry transfer apparatus (200 mA for 1 h). The membrane was blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 (v/v) and 5% powdered milk (w/v). Immunoreactive bands were detected with horseradish peroxidase conjugated goat, anti-rabbit IgG and detected by chemiluminescence using a high-sensitivity imaging system (LAS-1000; Fujifilm, Tokyo, Japan). The specificity of the two rabbit anti-myosin V antisera was documented by immunoblot analysis. Brain homogenates were prepared from normal, heterozygous (myosin V mid/) and myosin V-deficient, homozygous dilute mouse (myosin V mid/–). Both antibodies recognized a 190-kDa protein in the brain homogenates containing myosin V (heterozygotes) but showed no immunoreactive band in the homogenates of dilute mice, which lacks myosin V.

**Immunocytochemistry**—Astrocytes were seeded onto poly-d-lysine (10 μg/ml)-coated coverslips and grown for 24–48 h in growth medium. Medium was changed to serum-free DMEM ± 10 nM T₄ and treated with 1 μM Bt₂cAMP, 10 μM all-trans-retinoic acid, and 100 μM hydrocortisone for 16 h. Microtubules were depolymerized with 10 μM colchicine for 30 min before fixation. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then incubated with anti-myosin V antiserum (COOH terminus, 1:500), and the specific complexes were visualized by chemiluminescence and Kodak X-Omat AR5 radiographic film.

**Construction of Replication-deficient, Myosin V Viral Vectors**—The 3280-bp fragment containing the coding sequence of the globular myosin V tail cDNA (myosin V tail) was excised from clone D64 (a gift from Dr. Nancy Jenkins, National Cancer Institute, Frederick, MD) with SspI and Eco47III and ligated into the EcoRV site of the AdpREC shuttle vector. The shuttle construct was linearized with EcoRI and cotransfected with Xba-ClaI linearized Ad5-Pβgal into HEK 293 cells using Lipofect AMINE according to the manufacturer’s instructions. Replication-deficient Ad5-myosin V-containing virus particles were purified from the HEK-293 cell lysates by cesium chloride gradient centrifugation. Expression of myosin V from Ad5-myosin V-infected cells was confirmed by Western blot analysis. The Ad5-Pβgal virus particles were generated as detailed previously (20).

**Immunochemistry**—Astrocytes were seeded onto poly-d-lysine (10 μg/ml)-coated coverslips and grown for 24–48 h in growth medium. Medium was changed to serum-free DMEM ± 10 nM T₄ and treated with 1 μM Bt₂cAMP, 10 μM all-trans-retinoic acid, and 100 μM hydrocortisone for 16 h. Microtubules were depolymerized with 10 μM colchicine for 30 min before fixation. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then incubated with anti-myosin V antiserum (COOH terminus, 1:500), and immune complexes were visualized using a Texas Red-conjugated, anti-rabbit IgG. Images were collected by digital imaging microscopy in the Biomedical Imaging Facility at the University of Massachusetts Medical School. 20–50 random fields were examined per treatment group.

**Statistics**—All experiments were done a minimum of three times, and, where appropriate, statistical analysis was performed using Student’s t test.

**RESULTS**

**Myosin V Is Present in Astrocytes in Culture**—Myosin V comprises ~0.3% of total protein in brain. To determine the myosin V content in cultured astrocytes, we examined untreated and T₄-treated cells for the presence and distribution of myosin V using Western blot analysis and immunocytochemistry. As shown in Fig. 2A, ~90% of the 190-kDa, immunoreactive myosin V was found in the Triton-insoluble pellets prepared from retinoid-treated astrocytes in the absence or presence of T₄. Preincubation of the anti-myosin V antibody with excess blocking peptide (10 μg/ml) eliminated the 190-kDa immunoreactive band (see Fig. 1), indicating that the myosin V present in astrocytes is predominantly associated with the F-actin cytoskeleton. Also shown in Fig. 2A is the distribution of immunoreactive actin between the Triton supernatant and
myosin V is constitutively bound to F-actin. Fig. 2 shows that in the absence and presence of 10 nM T₄, no differences in total actin content were observed, and >90% of the immunoreactive actin was found in the Triton-insoluble pellet in both thyroid hormone-deficient and T₄-treated cells as determined by densitometry (data not shown). In control experiments, no specific immune complexes were observed in the 200-kDa range of immunoblots of astrocyte cell lysate, indicating that the anti-actin IgG did not cross-react with myosin V (data not shown). These data illustrate that, as reported previously (10), the retinoid-treated, thyroid hormone-deficient astrocytes contained a fully polymerized actin cytoskeleton and indicate that myosin V is constitutively bound to F-actin. Fig. 2B shows representative photomicrographs of the cellular distribution of immunoreactive myosin V in retinoid-treated astrocytes treated in the absence and presence of 10 nM T₄. Specific immunoreactive myosin V was found distributed throughout the cell in linear arrays and punctate clusters both in the absence and in the presence of T₄. These data show that astrocytes express abundant myosin V and that thyroid hormone does not affect the distribution of myosin V in the cell.

In Vitro Actin Binding Assay: Hormone-dependent Binding of p29 Vesicles to F-actin—T₄ specifically promotes the rapid redistribution of affinity-labeled p29 between the Triton-soluble and Triton-insoluble fractions in living cells (10). We exploited this to develop an in vitro binding assay to identify the components that mediate the hormone-dependent binding of the p29-containing vesicles to F-actin. Two different pools of astrocytes were prepared: one, the F-lysate, provided fully polymerized F-actin with its associated myosin V and was prepared by treating thyroid hormone-deficient astrocytes with 10 μM retinoic acid as described previously (10). The other, V-lysate, provided the affinity-labeled p29 vesicles in thyroid hormone-deficient astrocytes (5). Fig. 3 shows a representative fluorography of the effects of thyroid hormone on the distribution of affinity-labeled p29 vesicles in our in vitro actin-binding assay. As expected, comparable levels of affinity-labeled p29 were present in the lysate mixtures, as judged by the intensity of the lower band of the doublet of radiolabeled proteins at a region of ~30 kDa (21). In the absence of hormone, ~90% of the affinity-labeled p29 was found in the Triton supernatant. Addition of 10 nM T₃ to the mixed cell lysates had no effect on the distribution of affinity-labeled p29, because ~90% of the affinity-labeled p29 remained in the Triton-soluble fraction. In contrast, addition of 10 nM T₄ to the mixed cell lysates resulted in the binding of >70% of the affinity-labeled p29 to the Triton-insoluble, F-actin fraction. These data show that the T₄-dependent binding of the p29 vesicle to the actin cytoskeleton in a broken cell preparation mimics that observed in the living astrocyte (5).

The Binding of p29 to the Actin Cytoskeleton Is Calcium-, Magnesium-, and ATP-dependent—Although the in vitro actin binding assay showed that T₄ can initiate the binding of p29 endosomes to F-actin, whether this is a direct interaction between the vesicle and F-actin or is mediated by other actin-bound proteins, such as myosin V, remains to be established. A characteristic of myosin V is the ability to release the motor from F-actin by activating the Ca²⁺-dependent Mg-ATPase found in the actin binding head of myosin V (14, 22). We next examined whether activating the Ca²⁺-dependent Mg-ATPase would release p29 vesicles bound to F-actin. Equal volumes of F-lysate and V-lysate were mixed, and p29 vesicle:F-actin binding was initiated by adding 10 nM T₃ for 20 min. The reconstituted lysates were then treated with 0.1 mM Ca²⁺, 1 mM Mg²⁺, 90% of the affinity-labeled p29 was found in the Triton-insoluble fraction.
0.1 mM ATP, and/or 5 mM EGTA as indicated and incubated for an additional 30 min at 37 °C. Triton-insoluble pellets were separated from the Triton-soluble fraction, and the distribution of p29 was determined.

As shown in Fig. 4, ~80% of the total p29 vesicles added were bound to F-actin at the start of the experiment. Activation of Ca\(^{2+}\)-dependent Mg-ATPase(s) by the addition of divalent ions (Ca\(^{2+}\) and Mg\(^{2+}\)) and ATP resulted in the release of ~70% of the p29 vesicles from F-actin without altering the F-actin content in the Triton pellet. The calcium chelator, EGTA, blocked >50% of release of p29 from F-actin. Similarly, removing the substrate, ATP, or either divalent ion completely blocked the release of p29 vesicles from F-actin. These data suggest that myosin motor proteins, presumably myosin V, participate in the binding of the p29 vesicle to F-actin.

Characterization of the Interaction(s) between the Myosin V Tail and p29 Vesicles—Although the actin binding region of myosin V is located at the NH\(_2\) terminus of the protein, the vesicle binding region of myosin V appears to be located in the unique ~80-kDa globular tail (14). In the next series of studies, we generated truncation mutants of myosin V that lack the actin-binding head and examined the ability of these myosin V tail mutants to compete with the native, F-actin-bound myosin V for the p29 vesicles. Initial studies used the entire COOH terminus of myosin V synthesized in vitro from a 4.2-kb fragment (nucleotide 2911–7087) of the myosin V cDNA using the TNT-coupled transcription and translation system (TNT, Promega). Cell-free synthesis of the appropriate myosin V tail mutants to define the specific region(s) of myosin V tail that interact with the p29 vesicle. To simplify the analysis of the competition of the myosin V deletion mutations on T\(_4\)-dependent binding of p29 vesicles to F-actin, we modified the in vitro assay by replacing the affinity-labeled p29 with a GFP-tagged p29 fusion protein (p29\(^{\text{GFP}}\)) (5, 8). This allowed direct evaluation of the binding of fluorescent vesicles to F-actin without affinity labeling of the p29 or SDS-PAGE analysis. Exogenous p29\(^{\text{GFP}}\) was introduced into the astrocytes used to prepare the V-lysate by infection with replication-deficient Ad5-p29 GFP viruses. 48 hours after infection, >99% of the infected astrocytes expressed the p29\(^{\text{GFP}}\). Cells expressing the p29\(^{\text{GFP}}\) were then used to prepare the V-lysate as described under “Experimental Procedures”. Equal volumes of F-lysate and V-lysate containing p29\(^{\text{GFP}}\)-labeled vesicles were incubated with increasing concentrations of T\(_4\), rT\(_3\), or T\(_3\) (0–100 nm) for 20 min at 37 °C, and the quantity of p29\(^{\text{GFP}}\) in the Triton pellets was determined by fluorometry. Fig. 6 shows representative dose-response curves for thyroid hormone-dependent p29\(^{\text{GFP}}\) vesicle binding to F-actin. As expected, both T\(_3\) and rT\(_3\) initiated increases in the quantity of p29\(^{\text{GFP}}\) bound to the F-actin with EC\(_{50}\) values of ~1 nM, in close agreement to those reported previously (10). T\(_3\) had little, if any, effect on p29\(^{\text{GFP}}\) vesicle binding to F-actin, except for a modest 10–20% effect observed.
at 100 nM T₄, the highest concentration of hormone used.

To determine if the Δ myosin V tail was directly bound to the p29 vesicle, we introduced a Δ myosin Vtail into p29GFP-expressing astrocytes by infection with Ad5-ΔmyoVtail virus particles and examined the effects of T₄ on the binding of the myosin Vtail to immunopurified p29GFP vesicles. Cells were treated with or without 10 nM T₄ for 20 min, and the cell was lysed with 0.1% Triton. Vesicles containing the p29GFP in the clarified extract were immunoprecipitated by anti-GFP IgG (2 µg/ml), and those in the immunoprecipitated vesicles were resolved by SDS-PAGE. Shown in Fig. 7 is a representative immunoblot of Δ myosin Vtail-associated with affinity-purified vesicles from control p29GFP cells and from p29GFP cells expressing the Δ myosin Vtail. In control cells, no myosin V immunoreactive protein(s) was detected in the purified vesicle pool, because the native, F-actin bound myosin V was removed during clarification. In contrast, the Δ myosin Vtail showed a T₄-dependent association with the p29GFP vesicle, as judged by the co-purification of this 88-kDa immunoreactive band. These data show that the direct interaction between the p29 vesicle and myosin V is T₄-dependent.

In Vitro Analysis of the Effects of Myosin V Truncation Mutants on T₄-dependent p29 Binding to F-actin—Fig. 8 shows a schematic diagram of the myosin Vtail deletion mutations studied. All deletion mutants were synthesized by cell-free translation, and the synthesis of the correct myosin V polypeptide was confirmed by Western blot analysis (data not shown). The quantity of each mutant protein synthesized was determined by [³⁵S]methionine incorporation and ranged from 400 to 1000 ng/mg protein (data not shown). Individual Δ myosin V mutant proteins (~2–3 pmol of polypeptide/50 µl of mixture) were added to the actin-binding assay and preincubated for 20 min at 37 °C. T₄ (10 nM) was added, the mixtures were incubated for an additional 20 min, and the F-actin-bound, fluorescent p29GFP vesicles were then isolated in the Triton-insoluble pellet.

Data reported in Fig. 8 are expressed as the percentage of the maximum, T₄-dependent p29 vesicle binding observed in the absence of added competitors. Addition of the Δ myosin Vmid protein, corresponding to amino acids 504–1307, did not compete with native myosin V for the T₄-dependent binding of p29 vesicles to F-actin (p = NS). As observed above (see Fig. 5), addition of the entire myosin Vtail (residues 953–1852) de-
creased p29 binding to F-actin by >80% (p < 0.01). Addition of progressively shorter myosin V\(^\text{tail}\) deletion mutants: Δ myosin\(^{1513}\) (residues 1513–1852), Δ myosin\(^{1767}\) (residues 1767–1852), and Δ myosin\(^{1830}\) (residues 1830–1852) all competed against native myosin V and significantly decreased T\(_4\)-dependent p29 binding from 75% to 98% (p < 0.01). However, the Δ myosin V tail lacking only the last 44 residues (residues 953–1803) did not compete with native myosin V for T\(_4\)-dependent p29 vesicle binding. These data indicate that the T\(_4\)-dependent, vesicle-binding region of myosin V is located within the last 21 amino acids at the COOH terminus of the motor protein.

**Antibodies Directed Against the C Terminus of Myosin V**

Block the T\(_4\)-dependent Binding of p29 Vesicles to F-actin—Antibody inhibition studies were done to confirm the location of the vesicle-tethering region of myosin V. Two antibodies were used; one raised against the coiled-coil domain (residues 892–1040), and one directed against the COOH-terminal 21 amino acids (residues 1831–1852). The data summarized in Fig. 8 indicate that antibodies that are directed against the extreme COOH terminus of myosin V completely block the T\(_4\)-dependent binding of p29 vesicles to F-actin, whereas antibodies that are directed against the coiled-coil domain failed to alter p29 vesicle binding to F-actin. Control rabbit immunoglobulins had no effect of the T\(_4\)-dependent binding of p29 vesicles to myosin V (data not shown). These data confirm the assignment of the vesicle-tethering domain to the COOH terminus of myosin V.

Finally, we examined the possibility of a direct interaction between myosin V and T\(_4\). 2–3 pmol of cell-free-translated Δ myosin V\(^\text{tail}\) was incubated with 0.2 nM 125I-labeled 3\(_{-}\)-endocytotic vesicle-tethering domain to the COOH terminal 21 amino acids (residues 889–1040), and one directed against the COOH-terminal 21 amino acids (residues 1831–1852). The data summarized in Fig. 8 indicate that antibodies that are directed against the extreme COOH terminus of myosin V completely block the T\(_4\)-dependent binding of p29 vesicles to F-actin, whereas antibodies that are directed against the coiled-coil domain failed to alter p29 vesicle binding to F-actin. Control rabbit immunoglobulins had no effect of the T\(_4\)-dependent binding of p29 vesicles to myosin V (data not shown). These data confirm the assignment of the vesicle-tethering domain to the COOH terminus of myosin V.

**DISCUSSION**

In this study we show that a vesicle-tethering domain located at the COOH terminus of the unconventional myosin motor protein, myosin V, mediates the thyroid hormone-dependent attachment of endosomes to F-actin. The participation of myosin V in vesicle trafficking is well known (12, 14, 23). This motor protein has been shown to move vesicles along actin fibers in vitro (15, 24), and loss of myosin V in yeast and in mice leads to profound defects in cell migration, vesicle trafficking, and oriented organelle transport (25–27). In mice, dilute mutants show a lightened coat color and severe neurological defects that result in large part from aberrant vesicle transport and impaired cell migration, even though the actin cytoskeleton is unaffected (28, 29). In yeast, mutants of Myo2p, a class V myosin motor, show impaired vacuole transport and loss of polarized movement of membrane-limited organelles (27). The ability of myosin V to Dock with intracellular vesicles and tether these organelles to actin filaments is central to the function of this motor protein (30, 31) and also to the ability of myosin V to participate in the thyroid hormone-dependent endocytosis of D2 in brain.

Using a straightforward in vitro actin-binding assay, we showed that the binding of D2-containing endocytotic vesicles to F-actin was regulated by thyroid hormone. In situ, both T\(_4\) and T\(_3\), two transcriptionally inert thyroid hormones, initiate the actin-based endocytosis of the membrane-bound, short-lived, enzyme D2 (4, 5), mimicking events observed in brain in vivo (6). In vitro, the binding of D2 vesicles to F-actin showed the same iodothyronine specificity found in cAMP-stimulated astrocytes in situ (10) and in the brain in vivo (6). In CAMP-stimulated astrocytes, myosin V is tightly bound to the microfilaments, and activation of Ca\(^{2+}\)-dependent MgATPase(s), presumably the catalytic activity associated with the actin-binding head of the endogenous myosin V, released the D2-containing vesicles from F-actin. These findings are similar to those of others (14, 22) who found that addition of Ca\(^{2+}\), Mg\(^{2+}\), and ATP optimized the release of vesicles tethered to F-actin by myosin V, presumably by activating the ATPase. In contrast to the observations made with native, full-length myosin V, the Ca\(^{2+}\)-dependent MgATPase activity, associated with myosin V fragments lacking the neck and tail domains but retaining the actin binding head, is blocked by calcium and shows a different affinity for F-actin (32). Presumably, the loss of light chains and/or other accessory proteins accounts for the differences in the effects of Ca\(^{2+}\) on the interactions between the myosin V head and F-actin. Direct analysis of the interaction(s) between D2-containing endosomes and the ~88-kDa globular tail of myosin V showed that T\(_4\) promoted the direct binding of this polypeptide to the D2 vesicle.

Deletion analysis and antibody inhibition studies localized the vesicle-tethering domain to the COOH-terminal 21 amino acids of the motor protein. Using a series of progressively truncated myosin V\(^\text{tail}\) polypeptides that cannot bind to F-actin, the T\(_4\)-dependent binding of endosomes to F-actin was specifically blocked, presumably by competition with endogenous myosin V for the D2 vesicles. The demonstration of a direct T\(_4\)-dependent interaction between an exogenous myosin V\(^\text{tail}\) and the p29 endocytotic vesicle in situ indicates that the in vitro binding assay faithfully mimicked the events taking place in the cell.

Based on the results obtained with our actin-binding assay, the COOH terminus of myosin V appears to play a key role in the tethering of endocytotic vesicles. The globular tail region of the myosin V is highly conserved from fly to human (16). In mice, the loss of the COOH-terminal 13 amino acids leads to phenotypic defects in cargo binding (16, 33) that are similar, if not identical, to the loss of vesicle binding observed with our myosin V deletion mutants in vitro. Interestingly, mice homozygous for the Myo5a\(^{d-a}\) mutation that lack the last 14 amino acids of the myosin V and show neurological defects between days 14 to 21 of life that are similar to those of the dilute lethal mouse (33). Similarly, mice carrying the Myo5a\(^{d-a2}\) mutation that eliminates up to 92 amino acids from the myosin V tail, also show severe neurological defects during neonatal life; however, unlike the dilute lethal mouse, both the Myo5a\(^{d-a}\) and the Myo5a\(^{d-a2}\) mouse survive and show improved neurological function in adults. Such a delay in the developmental program of the brain is similar to the delays in brain maturation associated with neonatal hypothyroidism (34, 35).

Our studies show that myosin V plays a major role in the thyroid hormone-dependent, actin-based endocytosis of D2. In vivo, myosin V is abundant in nerve terminals in brain, where it associates with synaptic vesicles (23, 24, 36). Biochemical analysis showed that the synaptic vesicle proteins, synaptobrevin and synaptophysin, formed calcium-dependent complexes with myosin V (14), whereas ultrastructure analysis revealed that myosin V decorated the larger SV2-containing vesicles in the nerve terminal (15), i.e. a vesicle pool formed of recycling synaptic vesicles (16). These data suggest that myosin V plays a significant role in actin-based endocytosis of synaptic vesicles at the nerve terminal.

In CAMP-stimulated astrocytes, thyroid hormone-dependent endocytosis of the D2-containing vesicles does not require intact microtubules (4). Importantly, the interplay between microtubule-based and microfilament-based motors is central to the function of myosin V in brain. Although the microtubule motor kinesin mediates axonal translocation of myosin V and its associated vesicles to the nerve terminal (30, 37), the dy-
dynamic. T4-dependent regulation of D2 does not require intact microtubules, indicating that this is solely an actin-based endocytic event. Interestingly, two of the four myosin Vtail deletion mutants retain the kinesin binding AF-6 domain (38), and they were less effective competitors for D2 vesicles than deletion mutants lacking this domain. Because endogenous kinesins can interact with the AF-6 domain even in the absence of microtubules, it seems probable that the binding of kinesins to this domain on the MyoVtail and MyoV1513 mutants could partially mask the COOH-terminal tethering domain and thereby decrease their ability to compete for D2 vesicles. Elimination of the AF-6 domain produced deletion mutants that were very potent competitors for D2 vesicles. These data suggest that the vesicle-tethering domain is distinct from the AF-6 domain.

Although it is clear that myosin V tethers vesicles to the actin cytoskeleton in a T4-dependent manner, myosin V does not bind T4. These data suggest that at least one additional T4 binding protein is required and that its iodothyronine specificity and affinity are known. It is likely that this hormone-dependent linker protein is one of the accessory proteins bound to purified myosin V, because the interactions between detergent-insoluble lipid rafts and myosin V are mediated by protein complexes (39). On the other hand, vesicle-based docking protein(s) that expose a myosin V binding domain upon ligand binding is equally plausible. Importantly, our characterization of a specific region of myosin V used in this hormone-induced tethering event provides a powerful tool for identification and characterization of the putative, T4-binding docking protein.

In summary, we have shown that myosin V mediates the T4-dependent binding of primary endosomes to the actin cytoskeleton in preparation for internalization of the vesicle cargo. We also mapped the vesicle-binding region of myosin V to the most COOH-terminal part of the molecule. This is the first demonstration of a specific, hormone-regulated interaction between endocytic vesicles and an actin-based motor protein.

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