Real-time Visualization of Processive Myosin 5a-mediated Vesicle Movement in Living Astrocytes*S

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Recurrent endosomes in astrocytes show hormone-regulated, actin fiber-dependent delivery to the endosomal sorting pool. Recurrent vesicle trafficking was followed in real time using a fusion protein composed of green fluorescent protein coupled to the 29-kDa subunit of the short-lived, membrane-bound enzyme type 2 deiodinase. Primary endosomes budded from the plasma membrane and oscillated near the cell periphery for 1–4 min. The addition of thyroid hormone triggered the processive, centripetal movement of the recurrent vesicle in linear bursts at velocities of up to 200 nm/s. Vesicle migration was hormone-specific and blocked by inhibitors of actin polymerization and myosin ATPase. Domain mapping confirmed that the hormone-dependent vesicle-binding domain was located at the C terminus of the motor. In addition, the interruption of normal dimerization of native myosin 5a monomers inactivated the motor. In vitro experiments done in melanocytes limit the direct analysis of myosin 5a in living cells.

The trafficking of intracellular organelles utilizes both microtubules composed of polymerized tubulin and microfilaments composed of polymerized actin (1). Dyneins and kinesins provide bidirectional movement of organelles along individual microtubules, and members of the myosin superfamily use microfilaments for this function. Recent work done in melanocytes revealed that individual organelles can possess both microtubule- and microfilament-based motors and that the two cargo carrier systems cooperate in organelle trafficking (2, 3). Microtubule-based vesicle carriers are responsible for long range vesicle movement in these cells, whereas myosin 5a-mediated vesicle movement is restricted to short-range shuttling along the cortical actin cytoskeleton in the dendritic processes (4).

Four of the 18 identified classes of myosin motor proteins appear to transport vesicles along microfilaments in vitro (5), and loss of the two-headed myosin 5a motor disrupts vesicle trafficking in rodents and yeast (1, 2, 6–13). In melanocytes the myosin 5a null mouse, dilute, pigment granules remain centrally distributed and fail to accumulate in dendritic arbors (4, 14). The short distances traveled by cargo-laden myosin 5a in melanocytes limit the direct analysis of myosin 5a-mediated movement in living cells. In vitro, myosin 5a is a processive cargo-carrying motor and undergoes multiple catalytic cycles that allow the motor to move long distances along actin fiber tracks by taking large steps of ~36 nm (15–17). These estimates of the stepping size of myosin 5a closely agree with the distance between the two motor heads (18). The globular tail of the motor appears to bind tightly to the surface of melanosomes (4), synaptic vesicles (19, 20), and recycling vesicles in astrocytes (21). The tail of myosin 5a also shows hormone-regulated vesicle binding (21) and Ca2+-modulated interactions with two synaptic vesicle proteins, synaptophysin and synaptobrevin II, in isolated synaptosomes (22). However, the short distances traveled and the presence of cooperating microtubule-based cargo carriers have confounded the direct analysis of the processive movement and vesicle-docking reactions in living cells.

Astrocytes provide a unique model for the study of myosin 5a-mediated vesicle trafficking, because the dynamic hormone-dependent regulation of the short-lived membrane-bound enzyme type II deiodinase (D2)1 is an actin-based endocytotic event (21, 23–25) that results in the delivery of recycling vesicles from the cell periphery to the endosomal storage pool (26). In vitro, the C-terminal 22 residues of myosin 5a serve as the hormone-dependent vesicle-binding domain (21). This long range actin-based trafficking of recycling vesicles can be exploited to define the properties of myosin 5a-mediated vesicle movement in living cells without the participation of the microtubule cargo carriers.

In this report we use rapid acquisition time lapse microscopy to follow the processive, centripetal movement of individual recycling vesicles in living cells and show that a specific hormone(s) triggers the docking of primary endosomes to the C terminus of the actin-bound motor. Once tethered, the cargo-laden myosin 5a shows processive, centripetal movement from the cell periphery to the endosomal sorting pool with a velocity of ~100 nm/s. Actin depolymerization, myosin ATPase inhibitors, and the expression of motorless, dominant negative myosin 5a truncation mutants arrested the processive hormone-dependent movement of recycling vesicles. These findings provide the first evidence of the processive long range movement of cargo-laden myosin 5a along actin fibers in living cells and identify a specific thyroid hormone-dependent domain responsible for reversible vesicle docking.

1 The abbreviations used are: D2, type II iodothyronine deiodinase; BDM, 2,3-butanediol monoxide; D2p29, 29-kDa substrate binding subunit of D2; β-gal, β-galactosidase; GFP, green fluorescent protein; T₄, thyroxine; T₃, 3’,3,5-triiodothyronine; rT₃,3’,3’,5-triiodothyronine.
EXPERIMENTAL PROCEDURES

Materials—Thyroid hormones, Triton X-100, ATP, dibutyryl cAMP, hydrocortisone, colchicine, bovine serum albumin, 2,3-butanedione monoxime (BDM), and rabbit anti-actin IgG were obtained from Sigma. Dulbecco’s modified Eagle’s medium, antibiotics, Hank’s solution, and trypsin were purchased from Life Technologies, Inc. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). Anti-myosin 5a was generated as detailed previously (21). The anti-sera used were: anti-SV2 antisera (Dr. Kathleen Buckley, Harvard); anti-Synaptotagmin I and anti-Rab 3 A, B, and C antisera (Dr. Reinhard Jahn, Yale); and anti-GFP IgG (CLONTECH). Anti-D2p29 (27) and anti-p55 (44) (the subunit of protein disulfide isomerase) antibodies were raised in-house.

Culture Conditions—Astrocytes were prepared from 1-day-old neonatal rats as described previously (45) and grown in growth medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum, 50 units/ml penicillin, and 90 units/ml streptomycin. The 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.

Ad5-D2p29GFP Replication-deficient Adenovirus Construct—The D2p29GFP chimera was created as described previously (27). The replication-deficient Ad5-p29GFP virions were purified from HEK293 cell lysates by cesium chloride density centrifugation.

Dominant-negative Myosin 5a Mutants—The myosin 5a dimerization mutant, Δmyo5aHI-I, encodes the last IQ domain and the coiled-coil region (residues 892–1040, myosin 5a) was generated by reverse transcription-polymerase chain reaction using rat brain mRNA and site-specific 20-mer oligonucleotides (upstream 5′-CAGTGTGCTTCTCCGGCGGAT-3′; downstream, 5′-GTGAGGGCTTCCTTCACTTCA-3′). The ~500-base pair fragment was cloned into the EcoRV site of the prokaryotic expression vector, pTHioHis B (Invitrogen, San Diego, CA) and the Δmyo5aHI-I, DNA was then subcloned into the NotI site of the multiple cloning site of the Sinbis viral expression vector, pSinHis A (Invitrogen). The plasmid, containing a promoter for in vitro transcription as well as the Δmyo5aHI-I DNA, was linearized with PvuI, the mRNA was transcribed using InvitroScript CAP SP6 RNA polymerase. The transcript was transcribed-polymerase chain reaction using rat brain mRNA and site-directed mutagenesis (Invitrogen). The mRNA was collected 2–3 days post transcription.

The C terminus of myosin 5a (nucleotides 2911–7087, a gift from Dr. Nancy Jenkins) was cloned into the BamHI-NotI site of the multicloning site of Ad5AdpRec (27). The Δmyo5aΔHI-300 (nucleotides 5534–5560) containing the C-terminal-most 22 amino acids was cloned into the EcoRV site of the multicloning site of AdpRec. For both truncation constructs, the Δmyo5aΔHI-300 was linearized by EcoRI and cotransfected with CA1-XbaI linearized Ad5-Δ5-gal replicon-defective adeno virus DNA into HEK293 cells. The virus was propagated in HEK293 cells, and the cell lysates containing ~10⁶ plaque-forming units/μl replicon-defective Ad5-Δmyo5aΔHI-300 were then used as a reagent for in vivo analysis of D2p29 vesicle trafficking.

Rapid Acquisition Digital Imaging Microscopy—Rat astrocytes were grown on poly-L-lysine-coated (10 μg/ml) coverslips and infected with Ad5-D2p29GFP (multiplicity of infection 5–6), and expression of the GFP fusion protein was visually confirmed 24 h later. The cells were then grown in serum-free medium for 24 h, and D2p29GFP transfection to the plasma membrane was done by an overnight incubation with 100 nM hydrocortisone and 1 μM dibutyryl cAMP (45). Twenty minutes prior to study the microtubules were disassembled with 10 μM colchicine. Coverslips were then placed on a heated stage (37°C), the individual cells were isolated, and the treatment medium containing 10 nM thyroid hormone (T₄, T₃, or T₂) or 0.1% bovine serum albumin vehicle control was added. The movement of the D2p29GFP molecule was followed by collecting image data sets composed of 40 images taken at 250-nm focal increments. Each data set was collected in 200 msec and repeated every 15 s for a total of 10 min. Computer reconstructions (46) created three-dimensional images that were assembled into time-lapse movies. Representative QuickTime movies may be viewed in the on-line version (http://www.jbc.org).

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

RESULTS

Real-time Analysis of Hormone-dependent Vesicle Trafficking—Real-time rapid-acquisition digital microscopy was used to visualize the hormone-dependent centripetal movement of the recycling vesicles in astrocytes expressing a catalytically active green fluorescent fusion protein of the 29-kDa subunit of D2 (D2p29GFP) (27). Prior end-point analysis revealed that the principal circulating hormone T₄, but not the transcriptionally active metabolite T₃, initiated the delivery of D2 vesicles from the cell periphery to the perinuclear space over 20 min (23–25). D2p29GFP was delivered to the cell periphery prior to the vesicle trafficking study by cAMP stimulation, and the microtubules, which have no effect on recycling vesicle internalization, were disassembled with colchicine (23). Recycling vesicle movement was initiated by hormone(s), and the centripetal movement of the GFP reporter was monitored by collecting stacked image data sets at 250-nm focus increments through the cell every 15 s.

Three-dimensional image reconstructions were assembled into time-lapse movies, and the photomicrographs in Fig. 1 show representative “snapshots” of D2p29GFP-expressing cells at 0, 4, and 8 min after adding T₄, T₃, or no hormone. A two-dimensional projection of the three-dimensional data set (th1.ls) may be viewed in the on-line version (http://www.jbc.org). At the beginning of the experiment discrete D2p29GFP signals were distributed around the cell periphery in all treatment groups. In hormone-free cells, the fluorescent reporter wobbled in the same position on the plasma membrane throughout the experimental period. In T₄- and T₃-treated cells, the fusion protein remained at the periphery of the cell for up to 4 min and then showed episodic centripetal movement toward the cell nucleus. Individual vesicles in transit showed frequent perpendicular shifts preceded by short pauses, and by 8 min >80% of the recycling vesicles were deposited in the perinuclear space. In contrast, T₃-treated cells showed no directed movement, and the fluorescent reporter remained at the cell periphery as observed in hormone-free cells.
Myosin 5a-mediated Vesicle Movement in Astrocytes

Vector analysis and velocity diagrams of individual D2p29GFP vesicles in cultured rat astrocytes. Astrocytes expressing D2p29GFP were treated with no hormone or 10 nM T4, T3, or rT3 for 10 min. Individual D2p29GFP vesicles were followed over 10 min, and vector tracings (left) show the path of representative D2p29GFP vesicles during the 10-min test period. Starting points for mobile vesicles are identified as ●. The cell periphery and position of the nucleus (N) were outlined using Adobe Photoshop and included for reference. Velocity diagrams (right) were constructed for three representative recycling vesicles by measuring the distance traveled during each 15-s interval.

Vector analysis was then used to determine the path, distance traveled, and the velocity of 30–45 individual recycling vesicles. Fig. 2 shows the vector profiles for D2p29GFP vesicles in hormone-free and thyroid hormone-treated cells. In hormone-free cells or cells treated with T3, the D2p29GFP vesicles oscillated within 500 nm of the plasma membrane with average velocities (± S.E.) of 46 ± 8 nm/sec (n = 36, no hormone) and 40 ± 5 nm/sec (n = 30, T3-treated), respectively. In contrast, both T4- and rT3-treated cells showed centripetal but interrupted vesicle movement from the cell periphery to the perinuclear space. In both T4- and rT3 cells, individual vesicles showed 3–8 episodes of linear centripetal movement over the 10-min evaluation period, and the average of peak velocities (mean ± S.E.) during linear vesicle movement was 88 ± 16 nm/sec (n = 30) in the T4-treated cells and 105 ± 26 nm/sec (n = 32) in the rT3-treated cells.

T4-initiated Centripetal Vesicle Movement Uses Actin Cables—We next examined the effects of inhibitors of microfilament depolymerization and the myosin ATPase inhibitor, BDM, on recycling vesicle trafficking. Cyclic AMP-induced D2p29GFP-expressing astrocytes were pretreated for 20 min with 10 μM colchicine alone or in combination with either 10 μM dihydrocytochalasin B or 10 mM BDM, and vesicle movement was initiated by T4. As shown in Fig. 3, the colchicine-treated control cells showed that T4 initiated vesicle trafficking from the cell periphery to the perinuclear region of the astrocyte like that observed above (see Fig. 2). Consistent with earlier biochemical studies (21, 23), both 10 μM dihydrocytochalasin B and 10 mM BDM completely arrested T4-initiated vesicle movement (Fig. 3), and the vesicles remained within 500 nm of the cell periphery.

The C Terminus of Myosin 5a Tethers the Recycling Vesicle to the Actin-based Motor—Because our biochemical studies (21) showed that the C terminus of myosin 5a tethered the D2p29 vesicle to actin fibers in vitro, we examined the myosin 5a domain(s) responsible for the hormone-dependent docking of recycling vesicles in living cells. To deliver the recombinant myosin 5a tail mutants to astrocytes, we used replication-deficient adenoviral constructs containing the complete myosin 5a tail domain (Δmyo5atail), a fragment encoding for the last 22 residues of the myosin 5a (Δmyo5a1830), and a Sinbis pseudovirus containing the mRNA encoding the coiled-coil domain of myosin 5a (Δmyo5acoiled-coil). Control cells were infected with Ad5-β-gal to determine the influence, if any, of the adenoviral delivery system on astrocyte vesicle trafficking. Shown in Fig. 4 are representative vector tracings of individual D2p29GFP vesicles in T4-treated astrocytes expressing the individual myosin 5a truncation mutants and velocity diagrams for T4-initiated movement of individual vesicles. Representative video files for cells expressing the Δmyo5atail (T4_myo5atail.qt) and Δmyo5acoiled-coil (T4_myo5acoil.qt) may be viewed in the on-line version (http://www.jbc.org). All the Ad5-Δmyo5a mutant and Simbis-infected cells remained viable for up to 5 days after infection (the longest time tested) and showed cAMP-induced increases in D2 activity identical to that observed in uninfected cells. As expected, control cells expressing Ad5-β-gal showed T4-initiated centripetal vesicle trafficking identical to uninfected astrocytes, indicating that adenoviral infection did not alter hormone-dependent vesicle trafficking (Fig. 4). Overexpression of the Δmyo5atail completely blocked the T4-initiated vesicle transport and decreased the maximal velocity of D2p29GFP-containing vesicles to 38 ± 8 nm/sec (n = 30), a value equal to that in the hormone-free cell (see Fig. 2). Similarly, cells expressing the last 22 residues of myosin 5a (Δmyo5a1830) showed no hormone-dependent directed vesicle movement, and the D2p29GFP vesicles oscillated within 500 nm of the plasma membrane with average velocities (± S.E.) of 41 ± 5 nm/sec (n = 30). These data confirm that the last 22 residues of myosin 5a are essential for vesicle trafficking in situ and show that this domain serves as the hormone-dependent
vesicle-docking domain in living cells.

To determine whether myosin 5a-mediated vesicle trafficking in living cells required two functional motor domains, myosin 5a dimerization was disrupted with a myo5acoleiled-coil mutant (residues 892–1040), and the consequences on T4-initiated vesicle trafficking were examined. In cells expressing the myo5acoleiled-coil, D2p29 vesicle trafficking was divided into two roughly equal populations: one showed no directed movement (immobile), and the other showed directed long range centripetal movement identical to control T4-treated cells (mobile) (Fig. 4). Unlike our in vitro findings in which this 20-kDa myosin 5a mutant had no effect on vesicle binding to the actin-bound myosin 5a motor (21), in living cells it decreased the number of migrating vesicles by 50%. Mobile vesicles had peak velocities (± S.E.) during linear movement of 90 ± 10 nm/sec (n = 16), which is equal to that of cargo-laden native myosin 5a dimers (see Fig. 2).

Analysis of the individual vesicle paths in hormone-free cells, T4-treated cells, and the T4-treated cells expressing...
No hormone  T₄-treated  T₄ + Myo5acoil-coil

FIG. 5. Path and distance intervals for individual D2p29 vesicles in hormone-free, T₄-treated astrocytes, and T₄-treated astrocytes expressing myo5acoiled-coil. Cell images are redrawn from Figs. 2 and 4 for reference, and the paths of individual D2p29 vesicles are identified and processed as detailed under "Experimental Procedures." Individual vesicles originating at the plasma membrane (P) were followed throughout the experiment. The location of nucleus (N) is provided for reference. Bar = 1 μm.

Δmyo5acoiled-coil is shown in Fig. 5. In hormone-free cells, individual vesicles oscillated within 500 nm of the plasma membrane and did not migrate to the cell interior during the 10-min observation period. In T₄-treated cells, individual vesicles showed 3–8 episodes of linear centripetal movement of up to 3 μm that lasted up to 30 s with velocities ranging from 70 to 200 nm/s. All vesicles examined also showed several abrupt lateral shifts and occasional retrograde movement that bracketed the long range vesicle movements. Closer examination of individual members of the two pools of D2p29 vesicles in Δmyo5acoiled-coil-expressing cells showed vesicles with no directed movement similar to that in hormone-free cells (immobile vesicles) and vesicles with directed long episodic linear migration and cable shifts that are identical to those of D2p29 vesicles docked to the native myosin 5a dimers in T₄-treated cells (mobile vesicles) (Fig. 5).

Thyroid Hormone Initiates the Formation of a Vesicle-Myosin 5a Complex Without Affecting the Vesicle Budding Reaction—Both in vitro and in situ, T₄ initiates the formation of a detergent-insoluble complex between vesicles containing D2p29 and actin fibers (25, 26, 28). To determine whether the dominant negative Δmyo5a constructs altered the affinity of native myosin 5a for the actin cytoskeleton or competed with native myosin 5a for docking to the D2p29 containing vesicles, we examined the association of both native myosin 5a and the Δmyo5a constructs with the Triton-insoluble actin cytoskeleton (25, 26, 28). Because synaptophysin forms a reversible complex with myosin 5a in synaptic vesicles (22), we also examined whether this membrane-bound protein and selected other vesicle proteins were also part of this hormone-initiated actin-bound complex. Shown in Fig. 6A are the results of the immuno blot analysis of the T₄-initiated interactions between selected vesicle proteins, affinity-radiolabeled D2p29 (30), and native myosin 5a with the Triton-insoluble actin cytoskeleton. In cell lysates, T₄ treatment had little if any influence on any of the proteins evaluated, indicating that T₄ did not alter the formation of primary endosomes but promoted the formation of a complex between vesicle-docking protein(s) on the primary endosome and myosin 5a, presumably in preparation for centripetal movement of the vesicle to the cell interior. Rab3, synaptophysin, synaptotagmin, and D2p29 were all present in the Triton-insoluble complex of T₄-treated cells but lacking in this fraction from control hormone-free cells. In contrast, the full-length ~190-kDa myosin 5a was always found in the Triton-insoluble fraction from both hormone-free and T₄-treated astrocytes.

Direct analysis of the distribution of the native myosin 5a and the truncation mutants of myosin 5a is shown in Fig. 6B. Cells expressing Δmyo5acoil-coil, Δmyo5a1830, and Δmyo5acoiled-coil were treated with T₄ for 20 min, and the Triton-soluble and -insoluble fractions were prepared as detailed previously (25, 26, 28). As expected, the native myosin 5a was localized exclusively to the Triton-insoluble fraction of astrocytes expressing the Δmyo5acoil-coil, whereas the Δmyo5a1830 lacking the actin binding head was exclusively found in the Triton-soluble fraction. Similarly, >90% of the native 190-kDa myosin 5a in Δmyo5a1830-expressing cells was found in the Triton-insoluble fraction, suggesting that these two dominant negative myosin 5a constructs derived from the vesicle binding tail of myosin 5a do not alter the binding of native myosin 5a to the actin cytoskeleton.

In Δmyo5acoiled-coil-expressing cells, the native myosin 5a was found in both the detergent-soluble and detergent-insolu-
with the D2p29 vesicles in T4-treated D2p29 vesicles from Δmyo5a<sup>tail</sup>-expressing cells (Fig. 7B). In contrast, both the full-length native myosin 5a and the 20-kDa Δmyo5a<sup>coiled-coil</sup>-protein were found associated with D2p29 vesicles from cells expressing Δmyo5a<sup>coiled-coil</sup> indicating that Δmyo5a<sup>coiled-coil</sup>-native myosin 5a heterodimers can bind vesicles. These data confirm our in vitro findings that the vesicle-binding domain of myosin 5a is localized to the globular tail, specifically the last 21 amino acids of the motor protein in living cells. Further, they show that the influence of the dominant negative Δmyo5a tail mutants on T4-initiated vesicle trafficking depended on competition for vesicle binding sites rather than disruption of the binding of native myosin 5a dimers to the actin cytoskeleton.

DISCUSSION

The long range (up to 10 μm) myosin 5a-mediated vesicle movement in astrocytes differs from the more regionalized movement observed in cultured melanocytes. In Xenopus melanophores the long range transport of pigment vesicles relies on both the microtubule motor protein kinesin as well as myosin 5a (2, 31, 32). Actin depolymerization in fish melanophores prevents the stimulus-induced dispersal of melanosomes but not the long range microtubule-based movement of pigment granules (33). More recently, myosin 5a was shown to be responsible for the short range shuttling of pigment vesicles along filaments of the cortical actin cytoskeleton (1, 2, 4, 32, 34). Because the velocity of microtubule-based vesicle movement in dilute melanocytes is almost twice as fast as that in normal cells, it seems that vesicle-bound myosin 5a retards the kinesin-driven centrifugal progress by dynamic interactions with neighboring actin filaments alongside the microtubules (1, 4). Unlike the complex interactions between the microtubule- and microfilament-based motors observed in melanocytes, we show that long range centripetal movement of recycling vesicles in astrocytes is (i) mediated by myosin 5a, (ii) processive, (iii) hormone regulated, and (iv) unaffected by the microtubules or their associated motor proteins.

Time-lapse motion studies enabled us to examine the T4-initiated centripetal movement of recycling vesicles in living cells and to establish that myosin 5a delivered the vesicle cargo to the endosomal sorting pool along actin fibers. Immunoblot analysis of this recycling vesicle pool revealed that T4 promoted the formation of a complex of vesicle proteins with myosin 5a in situ without altering the formation of primary endosomes. Using a dominant negative experimental paradigm, we also found that blocking myosin 5a dimer formation partially arrested vesicle trafficking, whereas overexpression of the entire myosin 5a tail or the recently identified vesicle-binding domain (21) completely halted centripetal vesicle movement. These data identify a hormone-dependent vesicle-binding domain located within the last 22 residues of the myosin 5a motor protein and show that this region of the molecular motor is responsible for tethering the recycling vesicle in living astrocytes.

The hormone-dependent binding of recycling vesicles to myosin 5a in astrocytes is similar to the reversible interactions between several vesicle-docking proteins (35–37) and this motor protein (20, 22). A growing number of membrane-bound vesicle proteins that facilitate localization, docking, and degradation of the vesicle have been identified (35–38), and at least three of these vesicle proteins (39) form T4-dependent complexes with actin-bound myosin 5a in living astrocytes. Importantly, T4 does not alter the basic endocytic process, and the vesicle proteins Rab3, syntaptotagmin, and synaptophysin were all found in the detergent-resistant complex that presumably tethered the vesicle cargo to the actin-based motor.

The vesicle proteins that constitute this complex are fundamental to vesicle sorting in neurons and somatic cells. Rab3 is
a member of a group of GTP-binding proteins that target vesicles in transit to different intracellular destinations and along with synaptotagmin is found in recaptured synaptic vesicles in the presynaptic nerve terminal (39). Synaptophysin, the third vesicle protein that appears in the detergent-insoluble T4-detergent-insoluble V1 complex also forms a Ca\(^{2+}\)-modulated complex with myosin 5a in brain synaptosomes (22). Thus, the ability of T\(_{4}\) to promote interactions between key membrane vesicle-signaling proteins and myosin 5a is a potentially important function of this hormone that has been overlooked. Whether these three vesicle proteins are sufficient to form the docking complex for myosin 5a or other components are required remains to be established; no thyroid hormone binding domain has been reported on any of the members of this complex except for D2p29. However, it is unlikely that D2p29 initiates the formation of the docking complex, because alkylating substrate analogs covalently modify the T\(_{4}\) binding site without initiating actin-based endocytosis (30). This suggests that an as yet unidentified T\(_{4}\)-binding protein links the vesicle cargo to this binding domain of myosin 5a.

In vitro myosin 5a shows the properties of a processive motor protein and undergoes multiple ATP hydrolysis cycles coupled with mechanical displacement before dissociating from actin (16, 17). Rapid-acquisition digital image microscopy allowed us to determine the path taken, the distance traveled in a finite period, and the velocity of individual vesicles in living cells. Once captured by myosin 5a, individual vesicles showed episodic movement with a centripetal bias and a velocity of \(\sim 100\) \(\text{nm/sec}\), in close agreement with that determined for the short range movement of pigment-laden vesicles in melanocytes (4). Close inspection of the reconstructed time-lapse sequences revealed that vesicles showed frequent abrupt lateral shifts between episodes of linear movement. Such patterns of movement are observed for a single myosin 5a motor moving along actin fibers in vitro (16, 17) and are consistent with the ability of a processive motor to move between fiber track(s) in midstep.

As reported previously in vitro (21), expression of recombinant \(\Delta\)myosin5a\(^{1860}\) arrested hormone-initiated centripetal vesicle movement in living cells indicating that the recycling vesicles are tethered to myosin 5a through the last 22 residues of the motor protein. The myosin 5a tail domain has been implicated in vesicle transport from yeast to mice (8, 40). At least two spontaneously occurring mouse mutants have lost segments of the myosin 5a tail and show altered phenotypes that illustrate the consequences of disrupted myosin 5a vesicle trafficking. Mice homozygous for the dilute-neurological mutation (Myos5a\(^{d-d}\)) lack the last 14 residues of myosin 5a and show postnatal neurological defects that are similar to those of the dilute lethal mouse (8), in which the loss of this motor protein disrupts the synaptic vesicle cycle in maturing cerebellar granule neurons (20). Similarly, mice carrying the Myos5a\(^{d-d}\) mutation that eliminates the last 92 residues of the myosin V tail also show severe neurological defects during postnatal life. These defects in cerebellar development are reminiscent of the cerebellar defects and developmental delays in brain maturation that are often associated with neonatal hypothyroidism (41, 42), and the mapping of the neurological defects in dilute strains to the hormone-dependent vesicle-binding domain of myosin 5a provides an important clue to one of the molecular events that mediates the morphogenic effects of T\(_{4}\) in brain.

In vitro, myosin 5a monomers transport actin fibers (29, 43). Assembly of "crippled" myosin 5a motors composed of a native full-length monomer and a truncated mutant made from the coiled-coil dimerization domain decreased by \(\sim 50\%\) the number of vesicles showing hormone-dependent centripetal movement, suggesting that the loss of one motor head inactivates vesicle trafficking by myosin 5a. On the other hand, the remaining 50% of the recycling vesicles in cells expressing \(\Delta\)myosin5acoiled-coil showed centripetal movement with velocities, centripetal bias, and distances traveled that did not differ from control cells. These data suggest that a motor composed of two native myosin 5a monomers carried the mobile vesicles. Because no recycling vesicles were found that showed a slowed rate of travel, our findings in living cells also indicate that myosin 5a molecules with only a single motor domain are poor cargo carriers. This is consistent with the recent model of processive movement by myosin 5a proposed by Walker et al. (18). Alternatively, failure of the recycling vesicle to tether to the C terminus of the crippled myosin 5a would also reduce the number of mobile vesicles. The findings in living cells cannot distinguish between these two possibilities.

In summary we show that T\(_{4}\) initiates the tethering of recycling vesicles to the C terminus of myosin 5a and that the tethered vesicle moves down actin fiber cables to the endosomal sorting pool. Time-lapse sequences of reconstructed digital images allowed us to determine the velocity and distance traveled for individual recycling vesicles and to identify and characterize the hormone-dependent vesicle-binding domain of myosin 5a in a living cell. The ability to monitor the processive, centripetal movement of individual recycling vesicles in living astrocytes established the mechanism of myosin 5a vesicle trafficking in living cells and will prove invaluable for the characterization of the individual components that participate in the tethering of cargo vesicles to an actin-based motor protein.

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