Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 4, Issue of January 23, pp. 2333–2336, 2004
Printed in U.S.A.

Regulated Conformation of Myosin V*

Received for publication, November 10, 2003,
and in revised form, November 19, 2003
Published, JBC Papers in Press, November 22, 2003,
DOI 10.1074/jbc.C300488200

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We have found that myosin V, an important actin-based vesicle transporter, has a folded conformation that is coupled to inhibition of its enzymatic activity in the absence of cargo and Ca^{2+}. In the absence of Ca^{2+} where the actin-activated MgATPase activity is low, purified brain myosin V sediments in the analytical ultracentrifuge at 14 S as opposed to 11 S in the presence of Ca^{2+} where the activity is high. At high ionic strength it sediments at 10 S independent of Ca^{2+}, and its regulation is poor. These data are consistent with myosin V having a compact, inactive conformation in the absence of Ca^{2+} and an extended conformation in the presence of Ca^{2+} or high ionic strength. Electron microscopy reveals that in the absence of Ca^{2+} the heads and tail are both folded to give a triangular shape, very different from the extended appearance of myosin V at high ionic strength. A recombinant myosin V heavy meromyosin fragment that is missing the distal portion of the tail domain is not regulated by calcium and has only a small change in sedimentation coefficient, which is in the opposite direction to that seen with intact myosin V. Electron microscopy shows that its heads are extended even in the absence of calcium. These data suggest that interaction between the motor and cargo binding domains may be a general mechanism for shutting down motor protein activity and thereby regulating the active movement of vesicles in cells.

Mammalian myosin Va is involved in the transport of melanosomes, the pigment granules found in melanocytes, and secretory granules in neuronal cells (1–4). Its enzymatic and mechanical properties demonstrate that it is a processive motor, capable of taking many 35-nm steps per encounter with actin filaments (5–8). The 35-nm step coincides with the half-helical repeat of the actin filament and allows myosin V to effectively walk along one side of an actin filament (9).

The myosin V heavy chain is composed of four structural domains. The N-terminal motor domain possesses the actin and nucleotide binding sites of the molecule and is followed by a neck domain containing six IQ motifs which bind calmodulin (CaM)[1] (10). The proximal portion of the tail contains several segments of coiled-coil, driving the dimerization of two heavy chains, and the distal portion comprises a globular cargo binding domain. Electron micrographs of myosin V confirm that the molecule contains two elongated heads, a short rod and a bifurcated globular domain (11).

Tissue-purified myosin V requires micromolar Ca^{2+} for full MgATPase yet moves actin independent of the Ca^{2+} concentration in the *in vitro* assay (5, 11–13). Ca^{2+} also regulates the binding of myosin V to actin in the presence of ATP (14). However, recombinant myosin V heavy meromyosin-like fragments (HMM), which are missing the globular tail domain and the distal portion of the coiled-coil domain, have high actin-activated MgATPase rates in the absence of Ca^{2+} and are partially inhibited by Ca^{2+} if excess CaM is not present (10, 15), suggesting that the globular tail domain may be involved in down-regulation of enzymatic activity in the absence of Ca^{2+}.

Here we show that myosin V forms a compact structure in the absence of Ca^{2+} wherein the tail is folded and the heads fold back against it. This occurs under conditions where the enzymatic activity is largely turned off. A model is proposed to account for the regulation of vesicle trafficking in cells.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Myosin V was isolated from mouse brain and an HMM-like recombinant fragment of myosin V was expressed in Sf9 cells as described previously (10). The latter preparation contains some single-headed myosin V HMM molecules as seen by Coomassie Blue staining of SDS gels, coupled with Western blotting using an anti-FLAG antibody. Actin and CaM were prepared as described previously (16, 17).

Biochemical Assays—The actin-activated MgATPase activity was measured using an NADH-coupled assay (7), and the sliding actin *in vitro* motility assay (10) was described previously. The free Ca^{2+} concentration was calculated as described previously (18).

Analytical Ultracentrifugation—Sedimentation velocity runs were carried out at 20 °C and 50,000 rpm. Sedimentation boundaries were analyzed using time derivative analysis as described previously (19–21) using the recently developed software package Sedanal (22). A stock solution of myosin V or myosin V HMM was dialyzed overnight against buffer of 80 mM KCl, 20 mM MOPS (pH 7.4), 2.5 mM $MgCl_2$, 0.1 mM EGTA, 2 mM dithiothreitol. Just before running ATP (1 mM) was added, and when appropriate, $CaCl_2$ (0.2 mM) was added. Solutions in the range 0.1–1.0 mg/ml of protein were prepared using the dialysate as diluent. The density and viscosity calculated with Sednterp for the 80 mM KCl buffer were 1.00202 g/cc and 1.0013 cp, respectively, and for the 500 mM KCl buffer they were 1.0216 g/cc and 0.99592 cp, respectively. The value of the partial specific volumes for myosin and HMM were 0.742 and 0.741 cm^3/g, respectively, calculated from the amino acid composition using the consensus partial volumes of the amino acid residues (23).

Electron Microscopy—Myosin V was diluted to 80 nM protein, 80 mM (or 500 mM) KCl, 20 mM MOPS (pH 7.4), 2.5 mM $MgCl_2$, 10 μM ATP, 0.1 mM EGTA, 0.3 mM dithiothreitol, and 0.2 mM total Ca^{2+} when required

* This work was supported in part by Biotechnology and Biological Science Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CaM, calmodulin; HMM, heavy meromyosin; MOPS, 4-morpholinepropanesulfonic acid; cp, centapoise.

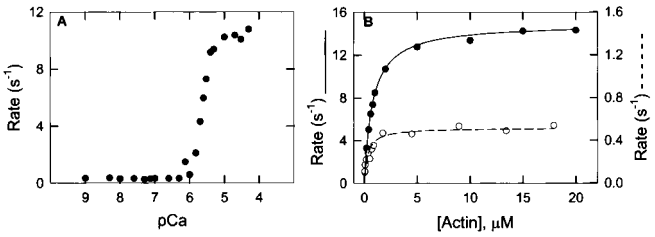

FIG. 1. **Effect of Ca^{2+} on the actin activated MgATPase activity of myosin V.** *A*, titration of the MgATPase activity with increasing free Ca^{2+} concentration. The conditions were 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 20 mM MOPS, 1 mM EGTA, 25 nM myosin V, 1 μM calmodulin, 10 μM F-actin, and varying $CaCl_2$ concentrations, 25 °C. *B*, actin titration of the MgATPase activity of myosin V in the presence (*solid circles* and *left ordinate*) or absence (*open circles* and *right ordinate*) of Ca^{2+}. Conditions were 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 20 mM MOPS (pH 7.0), 16 nM myosin V, 1 μM calmodulin, and either 0.1 mM EGTA or 0.1 mM EGTA and 0.15 mM $CaCl_2$, 25 °C. The V_{max} and K_{ATPase} were 14.7 s^{-1} and 0.75 μM in the presence of Ca^{2+} and 0.52 s^{-1} and 0.32 μM in the absence of Ca^{2+}, respectively. Note the different the scales on the *ordinates*. The activity in the presence of Ca^{2+} is on the *left ordinate* and that in the absence of Ca^{2+} is on the *right ordinate*. All rates are expressed per myosin V head per second. The *lines* are fits to the Michaelis-Menten equation.

at 20 °C. 5 μl was immediately applied to a UV-treated carbon-filmed electron microscope grid and stained with 1% uranyl acetate as described previously (9). Micrographs were recorded and processed as described (9) except that the 14.4-nm spacing of paramyosin filaments was used for calibrations.

RESULTS

Ca^{2+} Activates the MgATPase Activity of Myosin V—Similarly to previous results (12), we find that the actin-activated MgATPase activity of purified brain myosin V is strongly activated by the presence of micromolar Ca^{2+} (Fig. 1*A*). The lower activity in the absence of Ca^{2+} is due to an effect on the maximal ATPase activity, V_{max}, rather than on the apparent affinity for actin, K_{ATPase}, (Fig. 1*B*). Thus, the average steady state V_{max} is 12.6 ± 3.2 s^{-1} with a K_{ATPase} of 0.33 ± 0.2 μM in the presence of Ca^{2+} and 0.47 ± 0.1 s^{-1} with 0.41 ± 0.1 μM in the absence of Ca^{2+}. In marked contrast to intact myosin V, recombinant myosin V HMM shows a high actin-activated MgATPase even in the absence of Ca^{2+}, provided that 1 μM CaM is present in the assay buffer (10, 15) (Table I). Ca^{2+} also increases the MgATPase activity of myosin V in the absence of actin (Table I).

Ca^{2+} Induces a Large Conformational Change in Myosin V—To assess whether myosin V exhibits a Ca^{2+}-sensitive conformational change, we measured its S values in the analytical ultracentrifuge in the presence and absence of Ca^{2+} under ionic conditions similar to those used for ATPase measurements. In the absence of Ca^{2+}, myosin V sediments with an S value of 13.7 S (Fig. 2*A*, Table II). In the presence of Ca^{2+} this value reduces to 10.7 S. We will refer to these values as 14 and 11 S, respectively. Masses estimated using the sedimentation coefficient and diffusion coefficient during the runs show that the molecule is monomeric under both conditions. The shift in S value is consistent with myosin V adopting a more compact structure in the absence of Ca^{2+}, conditions where the ATPase is low, and changing to an extended structure in the presence of Ca^{2+}, conditions where the ATPase is high.

Under ionic conditions identical to those used for tissue purified myosin V, HMM underwent a smaller change from 8.5 S in the absence of Ca^{2+} to 9.4 S in its presence (Fig. 2*B*, Table II). Note that the direction of this change is opposite to that for intact myosin V and is consistent with HMM being more compact in the presence of Ca^{2+} than in its absence. Inclusion of 2 μM exogenous CaM in the buffer did not affect the sedimentation values for the HMM in either the presence or absence of Ca^{2+} suggesting that the effect is not merely due to dissociation of CaM in the presence of Ca^{2+} (Table II).

TABLE I
Effect of ionic strength on the regulation of the MgATPase activity of myosin V

Conditions: 10 μM actin, 2 mM $MgCl_2$, 1 mM ATP, 20 mM MOPS (pH 7.0), 1 mM phosphoenolpyruvate, 0.2 mM NADH, 40 units/ml lactate dehydrogenase, 200 units/ml pyruvate kinase, and either 0.1 mM EGTA or 0.1 mM EGTA plus 0.2 mM $CaCl_2$. The KCl concentration is as indicated. The numbers are the average of two independent experiments of different preparations of myosin V.

[KCl]	MgATPase activity			
	Myosin alone		Actomyosin	
	$-Ca^{2+}$	$+Ca^{2+}$	Ca^{2+}	$+Ca^{2+}$
	s^{-1}		s^{-1}	
50 mM	0.029	0.080	0.95	9.62
500 mM	0.063	0.064	2.20	1.33

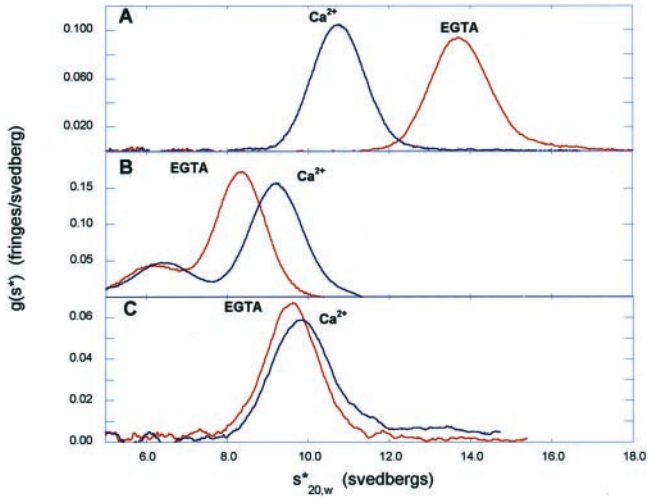

FIG. 2. **Effect of Ca^{2+} on the sedimentation properties of myosin V and myosin V HMM.** In each case the blue curve represents the data in the presence of Ca^{2+} and the red curve is the data in the absence of Ca^{2+}. *A*, sedimentation velocity profiles of myosin V in 80 mM KCl. *B*, sedimentation velocity profiles of myosin V HMM in 80 mM KCl. The material sedimenting at about 6 S is likely to be single-headed myosin V HMM. Interestingly, it undergoes even less of a Ca^{2+}-dependent shape change than does the two-headed HMM. *C*, sedimentation velocity profiles of myosin V in 500 mM KCl. Conditions were either 500 or 80 mM KCl (as indicated), 20 mM MOPS (pH 7.0), 2.5 mM $MgCl_2$, 1 mM ATP, and either 0.1 mM EGTA or 0.1 mM EGTA plus 0.2 mM $CaCl_2$, 25 °C.

TABLE II
Effect of ionic strength and Ca^{2+} on the sedimentation coefficients of intact myosin V and myosin V HMM

Conditions are as described under "Experimental Procedures" except for the HMM + CaM sample, which also contained 2 μM CaM.

	Sedimentation coefficients (s_{20})		
	[KCl]	Ca^{2+}	EGTA
Myosin V	80 mM	10.7	13.7
MyosinV	500 mM	9.8	9.6
HMM	80 mM	9.4	8.5
HMM + CaM	80 mM	9.4	8.5

A Compact Structural State for Myosin V—Myosin V was viewed in the electron microscope following negative staining. In the absence of Ca^{2+} (at 80 mM KCl), the majority of the molecules on the grid showed a novel, compact triangular shape (Fig. 3*a*). Single particle image processing reveals an enigmatic but rather consistent substructure (Fig. 3*b*) in which both the 32-nm heads and 57-nm tail must be folded because the lengths of the sides of the triangle (22–27 nm in the image averages) are shorter than either of these domains (compare

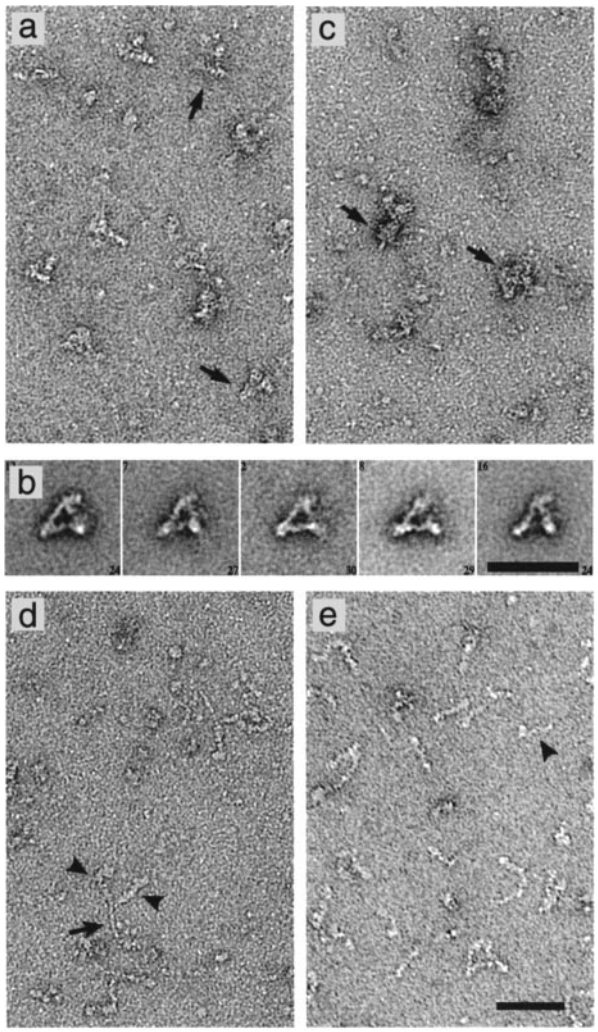

FIG. 3. **Negative stain electron microscopy of myosin V.** *a*, low Ca^{2+}, low ionic strength; *arrows* indicate two examples of the compact molecules visible in this field. *b*, typical averaged images from single particle image processing of 387 compact molecules; class sizes are about 30 molecules. *c*, field of myosin V in 0.1 mM free Ca^{2+}; structures seen (*arrows*) are very variable. *d*, high ionic strength, low Ca^{2+}; molecules are not compact. Instead, the two heads (*arrowheads*) and tail (*arrow*) of molecules are visible. *e*, myosin V HMM at low Ca^{2+}, low ionic strength; some single heads are also present (*arrowhead*) in agreement with the ultracentrifugation results (Fig. 2*B*). *Scale bars*, 50 nm. *Bar* in *panel e* is for *panels a*, *c*, *d*, and *e*.

with Fig. 3*d*), and the tail domains are not obvious. We cannot yet confidently assign the features of the compact structure to substructural domains of myosin V. These molecules are markedly different from ones stained at 0.5 M KCl in the absence of Ca^{2+}, under similar conditions to those used previously to produce freeze etch, rotary shadowed images of intact myosin V (11). Here the molecules adopt the familiar T- or Y-shape, with two readily identifiable elongated heads coupled to the tail (Fig. 3*d*). Correspondingly, the sedimentation coefficient of intact myosin V at 0.5 M KCl in the absence of Ca^{2+} is reduced to 9.6 S (Fig. 2*C*), very similar to that found at low ionic strength in the presence of Ca^{2+} (Fig. 2*A*). Ca^{2+} induces a very small change in S value (to 9.8 S) at high ionic strength (Fig. 2*C*, Table II). The actin-activated MgATPase activity at high ionic strength is not regulated (Table I). Therefore, purified myosin V at high ionic strength behaves much like HMM at low ionic strength. It has an extended structure in the electron microscope, like HMM (Fig. 3*e*), and its sedimentation coefficient undergoes a modest Ca^{2+}-induced shift in the same direction as HMM (Fig. 2*B*).

When purified myosin V or HMM was viewed at low ionic strength in the presence of Ca^{2+}, few identifiable structures were observed whether or not 1 μM exogenous CaM was present (Fig. 3*c*). We believe this is due to either full or partial dissociation of individual CaM light chains from their IQ motif binding sites which would allow the levers to be more flexible and adopt very diverse shapes. Myosin II light chain dissociation has previously been shown to produce a more compact lever arm (24). In the presence of 1 μM exogenous CaM, chelation of the Ca^{2+} by 5 mM EGTA reverses the instability of myosin V over a period of 30 min so that the compact appearance is restored, and on subsequent addition of salt the extended conformation is also seen.

DISCUSSION

We have shown that myosin V adopts a compact folded conformation with a sedimentation coefficient of 14 S under *in vitro* conditions where the actin-activated MgATPase activity is turned off. The reason this structure has not been seen before is that the high ionic strength conditions used in earlier microscopy favor the extended form. Several interesting questions arise. First, how does myosin V translocate cargo in cells where the Ca^{2+} levels are typically less than one micromolar, and second, how is the lack of regulation seen in *in vitro* motility assays reconciled with the tight regulation observed in MgATPase assays? These questions may be linked. We postulate that the binding of myosin V to its docker or linker protein unlocks the folded conformation and results in an active myosin even in the absence of Ca^{2+}. For example, myosin V in mammalian melanocytes binds via its tail domain to the C terminus of melanophilin, which, in turn, binds via its N terminus in a GTP-dependent manner to Rab27a to make an active tripartite complex (25–27). The tail of myosin V is alternatively spliced and the melanocyte specific isoform has an exon required for binding of melanophilin that is not present in the brain myosin V isoform we isolated in this study (25). We predict that addition of melanophilin to the melanocyte-specific isoform but not to the brain isoform triggers the extended active conformation even in the absence of Ca^{2+}. It is likely that other myosin V isoforms have similar linker proteins that would act in an analogous manner. It is possible that binding of myosin V to a coverslip surface, *in vitro*, triggers the same conformational change in myosin V, leading to an active molecule. This would explain why myosin V is always active in assays where it is bound to a surface.

An advantage of having a highly regulated myosin V is that unregulated, non-cargo-bound myosin V in cells would needlessly hydrolyze ATP and would stay associated with actin filaments due to its processivity. This behavior would be even more pronounced in elongated cells such as neurons and melanocytes where myosin V participates in the transport of vesicles to the dendritic tips for docking with the cell membrane. If the activity of the molecule were unregulated myosin V would not be available for newly formed vesicles or cargo in the cell body. If, on the other hand, myosin V could dissociate from the vesicle after docking and fold into an inactive form that could freely diffuse, it could then be effectively recycled.

We propose a model whereby free myosin V in cells under conditions of low Ca^{2+} concentration exists in the 14 S conformation and is enzymatically and mechanically inactive (Fig. 4). The binding of cargo to the tail region occurs either directly or indirectly via linker proteins and triggers myosin V to adopt the extended, active conformation. This interaction might be regulated by phosphorylation of myosin V or any of the linker proteins or by the nucleotide status of a G-protein that is part of the linker complex. The change to the extended conformation is also triggered directly by Ca^{2+} binding to myosin V, but this

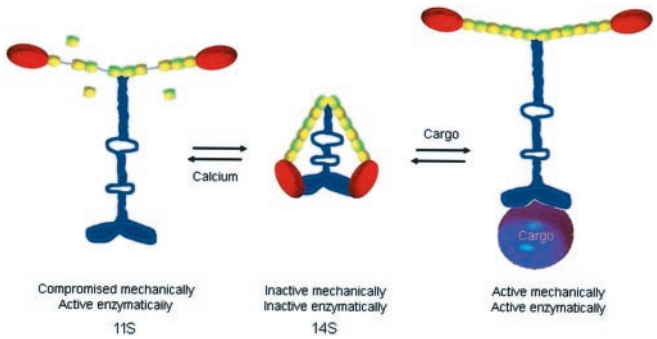

FIG. 4. **Speculative model for conformation-dependent regulation.** Under normal low Ca^{2+} conditions in cells, myosin V would be either bound to cargo or folded into an inactive form that would not move on actin filaments. Regulation of cargo binding to myosin V either directly or via linker proteins might be regulated by the state of phosphorylation of the myosin V cargo binding domain, phosphorylation of the linker protein, or by the nucleotide state of G-proteins. Increases in cellular Ca^{2+} levels might elevate the actin-activated MgATPase activity, but if CaM molecules dissociate, little mechanical activity would be produced. The schematic is not to scale and the depiction of the 14 S conformation is speculative.

may result in a mechanically weakened protein, since some CaM molecules dissociate from myosin V in the presence of Ca^{2+} (15, 28). Myosin II stripped of one of its light chains retains its actin activated MgATPase activity but performs poorly in mechanical assays such as the *in vitro* motility assay or step size measurements using the optical trap (29–31). Thus, even though myosin V adopts the 11 S extended conformation in the presence of Ca^{2+}, it may not be a fully functional motor under these conditions.

Regulation of motor protein activity by a tail-dependent conformational change plays a role in the regulation of conventional kinesin (32–34) and has been adopted by at least two myosin family members (II and V). In addition, there is indirect evidence for a role of the tail in the regulation of a mammalian myosin I (35). The structural basis for the regulation of myosin V is very different from that of smooth muscle myosin II, however. The enzymatic activities of smooth and nonmuscle HMM are regulated by phosphorylation even though these molecules are missing much of their tails, whereas that of myosin V HMM is unregulated (36, 37). Thus, the compact folded structure observed with smooth and nonmuscle myosins may be more important for regulation of filament assembly than for enzymatic activity. A particular asymmetric interaction between the two heads of smooth muscle HMM is seen in the off state that may be important for the enzymatic regulation (38). The compact form of myosin V also has a specific structure, but it differs in having the globular tail domains incorporated within the head region. The fact that the microtubule-dependent motor, kinesin, and two myosin family proteins are now known to have evolved regulatory mechanisms involving head-tail interactions that occur via large conformational changes is very significant. It suggests that future studies testing for motor molecule regulation should examine the enzymatic properties of complete molecules, along with any associated subunits. In kinesin the portion of the globular tail domain responsible for the inhibition has been identified (33). The next challenges with myosin V will be to identify the putative interacting portions of the head and tail and to determine how these interactions are regulated and how they are disrupted by cargo binding.

Acknowledgments—We thank Robert Adelstein, Peter Bayley, Stan Burgess, Mihály Kovács, Judit Tóth, and John Trinick for helpful discussions; Matt Walker and Chun Feng Song for contributing to the microscopy; and Estelle V. Harvey for her excellent technical assistance.

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