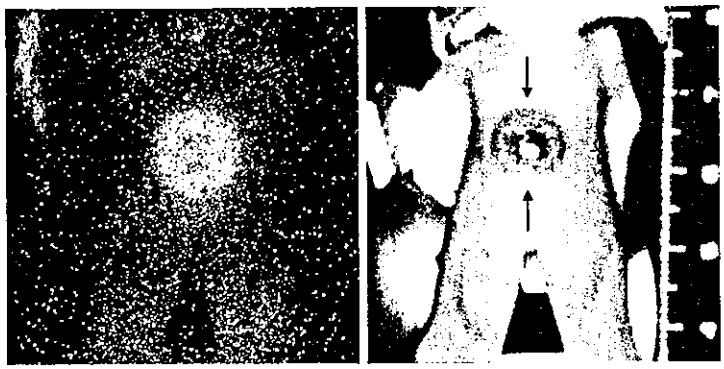

Biophoton Image Photograph Sample Slice with HE Stain

Fig. 4. Comparisons between biophoton image, raw image** and pathological findings of AH109A. An ultraweak biophoton image, raw image and histological images at week 1. The arrows drawn on the tumor corresponds to the cross-sectional line. ** The raw image taken under very weak illumination (3000–5000 photon counts/s) with the CCD camera used for the biophoton imagery.

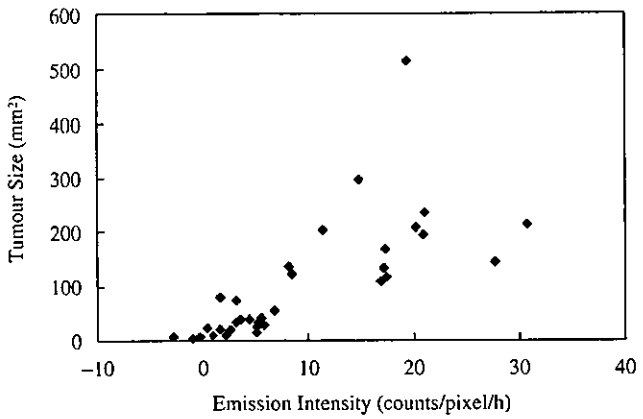

Fig. 5. Correlation between biophoton intensity and square root of tumor size at week 1. Tumor size and ultraweak biophoton intensity at the first week are plotted in the graph. The correlation coefficient of the tumor size at week 1 and ultraweak biophoton intensity is 0.73.

Results

Tumor growth and biophoton imaging. The tumors exhibited different growth modes for each cell line. Representative growth time courses and biophoton images are shown in Fig. 2. The rat hepatoma cell line AH109A showed the most rapid growth, reaching a size of about 700 mm² in 3 weeks. The human esophageal carcinoma cell line TE4 grew to the size of about 100 mm² in 3 weeks and the human esophageal carcinoma cell line TE9 grew to the size of about 40 mm² in a week, but shrank thereafter. The size of TE4 at week 1 was 24 mm².

The biophoton patterns also showed differences in each tumor cell strain. Although AH109A exhibited a homogeneous biophoton pattern at week 1, it exhibited a heterogeneous pattern after 2 weeks (Fig. 2). The emission intensity from TE4 was rather weak in the first week, but became more intense in weeks 2 and 3. The biophoton pattern from TE4 showed homogeneity until the third week. In contrast, TE9 showed very weak light emission; tumors were recognized as arising from the lower biophoton intensity area compared to neighboring normal tissue throughout the measurement period.

Nude mice showed specific biophoton emission related to internal organs and muscles in all measurements.

Comparisons of the biophoton pattern and surface appearance of the tumors showed distinct differences. In Fig. 3, part of the tumor showed high-intensity biophoton emission despite being covered with necrotic skin. In another area, the tumor showed low-intensity biophoton emission despite being covered with normal skin. Pathological findings suggest the area with high-intensity biophoton emission contains live tissue and the area with low-intensity biophoton emission contains necrotic tissue (Fig. 3).

Table 1. Comparison of tissue viability and biophoton intensity of AH109A

Tissue viability	Biophoton intensity	
Live tissue	2.93±3.34[1)]	[2)]
Necrotic tissue	10.76±6.52[1)]	

1) Photon counts/pixel/h.
2) P=2.30E-8.

Biophoton intensity and tumor growth rate. Pathological observation of AH109A at week 1 revealed homogeneous tissue (Fig. 4).

The average biophoton intensities of the TE9, TE4 and AH109A tumors at week 1 were 3.37±2.23, 2.42±3.09 and 13.55±8.40 counts/pixel/h, respectively. After 1 week, AH109A reached 167.1±55.1 mm², while TE9 grew to 41.4±18.7 mm² and TE4 grew to 24.1±12.7 mm². AH109A showed the highest emission intensity among all the tumors. The biophoton intensity of AH109A was significantly higher than those of TE9 and TE4 (P=0.0001 and P=0.00006, respectively). The P value between TE4 and TE9 was 0.49.

The relationship between biophoton intensity and tumor size at week 1 is shown in Fig. 5. The correlation coefficient of the biophoton intensity and tumor size at week 1 for all tumors was 0.73 and the correlation coefficient of the biophoton intensity and the square root of tumor size was 0.82.

Biophoton intensity and tumor viability. The emission intensity of living tissue, measured in 40 areas, amounted to 10.76±6.52 photon counts/pixel/h, and that of necrotic tissue measured in 26 areas was 2.93±3.34 photon counts/pixel/h. The P value was 0.00077 (Table 1).

Discussion

Although the biophoton images of the AH109A tumor exhibited homogeneous patterns at week 1, they exhibited heterogeneous patterns thereafter. This heterogeneity is not due to the surface condition of the skin, but reflects the viability of the un-

derlying tumor tissue, as judged by microscopic observation.

TE4 grew to approximately 100 mm^2 in 3 weeks and the biophoton image exhibited a homogeneous pattern until the third week. Pathological findings of TE4 showed a homogeneous pattern in accord with the biophoton image.

The biophoton intensity of TE9 at week 1 was greater than that of TE4. TE9 exhibited an extremely weak biophoton intensity at week 3 that was lower than the emission intensity of TE4 or the normal tissue around the tumor. The growth pattern of TE9, i.e., reaching maximum size during week 1 and shrinking thereafter, is in agreement with the biophoton results. Pathological findings showed no vessel formation in TE9.

Although the biophoton images of TE4 and TE9 at week 1 cannot be easily differentiated from those of normal tissue at present, spectral analysis may be helpful for recognition of such tumors.[11]

The non-tumor regions in the nude mice showed specific biophoton patterns related to the organ distribution. The lumbar muscle and digestive organs showed enhanced biophoton emission. These phenomena suggest that studies to evaluate the relationship between physiological function and biophoton emission would be of value.

The growth rates and biophoton intensities of AH109A tumors are significantly higher than those of TE9 and TE4. Additionally, biophoton intensity and tumor size at week 1 were correlated, with a correlation coefficient of 0.73 (Fig. 5). This result suggests that ultraweak biophoton emission is related to growth activity, presumably via metabolic activity. Thus, we can argue that areas with high intensity of biophoton emission contain live tissue and have high-growth activity, while areas with low-intensity biophoton emission contain necrotic tissue or tissue with a very low-growth rate.

Biophoton emission has been attributed to oxidative metabolism in live organisms, and it was reported that electron leakage from mitochondria results in the generation of active oxygen species such as the superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen.[16] Oxidation of cellular molecules causes excitement of other fluorescent molecules that results in biophoton emission.[1,17-19] In addition, cancer tissue contains fewer reactive oxygen quenchers than normal tissues, including superoxide dismutase (SOD) and catalase.[20] In normal tissue, reactive oxygen species are immediately eliminated by self-defence mechanisms consisting of SOD, catalase, vitamin E, glutathione, etc., in the cells. In morbid tissue, however, the balance between reactive oxygen generation and quenching activity is destroyed. Although the mechanism of this phenomenon is not clear at present, it is speculated that the relatively fast growth of malignant tumor generates a large amount of reactive oxygen species, leading to intense biophoton emission. The mechanism of this phenomenon should be examined.

Inflammation is also a cause of biophoton emission owing to active oxygen species from neutrophils, which generate singlet oxygen. Since singlet oxygen shows specific absorption at 703 nm, spectral analysis might be useful to distinguish malignant tissue from inflammation.

Tumor growth rate is one of the most important factors that define malignancy. No existing imaging modality except for positron emission tomography (PET) is able to evaluate metabolic activity. Our results suggest that biophoton measurement can detect growth activity, and it requires no isotope-labeled substrates, nor a cyclotron to prepare them, as is needed for PET. Biophoton measurement requires only a completely shielded space and compact detectors, requiring no chemical administration. Thus, biophoton measurement could be useful as a simple non-invasive method to obtain pathological information. PET is preferably used in screening of distant metastases or occult lesions of malignancies. Applications of biophoton measurement should be different from those of PET, because biophoton emission at the body surface represents light emission from only as deep as 3 mm from the tissue surface. Thus, biophoton measurement might be suitable for non-invasive sequential or repeated pathological diagnoses of recognized tumors but not for screening. For measurement of deeper tissue, a needle-coupled measurement probe would be needed. Biophoton measurements also have the advantage of being inexpensive.

This measurement technique may also be available for recognizing extremely thin tumors that are not palpable or visually apparent.

Image aquisition took 1 h in this study, and the measurement time should be shortened for clinical application. Recent development of measurement apparatus with high efficiency can shorten the image acquisition time, which is less than 30 min with the latest detector.

Biophoton images obtained from growing tumors can provide information about tumor properties, including whether the tumor is alive or not and how fast it is growing. In addition, biophoton images reveal tumor viability even when the surface skin is necrotic.

The effectiveness of chemotherapy is generally assessed in terms of tumor size. However, this standard parameter often shows a slow response after application of anticancer therapies, because the reduction of tumor size usually occurs much later than tissue necrosis. Therefore real-time estimation is impossible using this parameter. Biophoton measurements might allow real-time assessment of tumor viability through detection of changes in emission intensity.

In our study, necrotic areas showed low emission intensity in the heterogeneous mass. These results suggest that we can distinguish living area and necrotic area by biophoton measurement. Moreover, sequential measurement of biophoton emission during chemotherapy may enable us to detect tumor necrosis induced by chemotherapy. Because different chemotherapeutic agents have distinct mechanisms of action, characteristic changes of biophoton emission during the transition from the living state of the tumor to the necrotic state might be observable.

In conclusion, this method could be useful to assess not only malignancy, but also the efficacy of chemotherapy or radiotherapy in terms of viability, rather than tumor size. Although biophoton emission can be detected only on the body surface at present, deeper targets should be detected by the use of endoscopy or needle-coupled devices. This procedure may thus provide a non-invasive or minimally invasive optical biopsy as an adjunct to or replacement of existing diagnostic methods. We are now trying to detect other malignant xenografts to confirm the validity of this approach to measure tumor growth rate.

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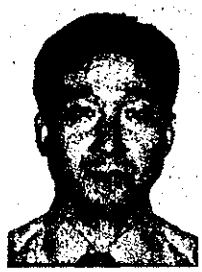
6 ナノセンシングカプセルの医療応用と未来展望

■武田元博（たけだもとひろ） 東北大学大学院医学系研究科外科病態学講座
粕谷厚生（かすやあつお） 東北大学加齢医学研究所
川添良幸（かわぞえよしゆき） 東北大学学際科学研究センター
大内憲明（おおうちのりあき） 東北大学大学院医学系研究科外科病態学講座



武田　元博
1987年東北大学医学部医学科卒業。89年東北大学第二外科入局。93年山形県テクノポリス財団主任研究員。2001年東北大学病院乳腺分泌外科助手。
研究テーマは乳腺外科，ナノテクノロジー，生物フォトン現象。趣味は登山，車いじり，料理。

Key words: nano-sized sensing capsules, fullerene,Oligo-dendrimer, silicone nano-cluster peptide-nano carrier

Abstract

近年のナノテクノロジーの進歩は医療に影響を与えつつある。真のナノテクノロジーは従来のマイクロマシーン技術を小さくすることではなく、分子設計に基づき、原子から新たな分子・物質を創造することである。例えば従来存在しないと考えられてきたフラーレンタイプのシリコンクラスターは、2001年Kawazoeらにより安定な構造を取り得ることが初めて予測された。その翌年Oharaらによりその存在が実証され、その蛍光特性から新たな医療応用が期待される。このように新たな物質が次々に創生されつつあり、医療技術に大きな変化をもたらすと考えられる。

はじめに

現在，シリコン加工技術はサブミクロンレベルに達し，最近ではさらに小さなレベルでの制御技術による新た物質の創生が注目されている。これは，従来のサイズを小さくして行く技術ではなく，原子·分子の特性を基礎とし，その組合せによって，指先に乗るチップ中に図書館全体の所蔵物を書き込めるような超高密度デバイスなどを実現する技術といい換えることができる。ナノテクノロジーは，このように未だ実現していないナノメートルレベルの物質を扱う，将来飛躍的に発展するであろう高度医療技術社会に対応していくための技術でもある。ナノテクノロジーによって創生された物質としてカーボンフラーレン，シリコンフラーレンなどが挙げられるが，この創生には近年のコンピュータサイエンスの飛躍的な発展が大きく関与している。すなわちクーロン相互作用する電子と原子核からなる多体系に対する量子力学の方程式を数値的に解く，第一原理計算と呼ばれるスーパーコンピュータを用いたシミュレーションにより，実験以前に新物質の構造と物性を予言するなどの技術が急速に発展した。コンピュータサイエンスの発展の結果，従来予測し得なかった構造・物理的性質を持つ分子やクラスター構造が今後次々と予測・作製されると考えられる。本稿では近年実用化しつつある，機能性クラスター＝ナノディバイスの医療応用について解説する。

1. ナノクラスター

近年，分子レベルで有用な機能を備えるさまざまな構造が発見され，その応用が始まっ

Medical Application and Future Innovation of Nano-sized Sensing Capsules: Motohiro Takeda, Noriaki Ohuchi, Graduate School of Medicine, Tohoku University,Kousei Kasuya, Institute for Differentiation, Aging and Cancer, Tohoku University, Yoshiyuki Kawazoe, Center for Interdisciplinary Research, Tohoku University

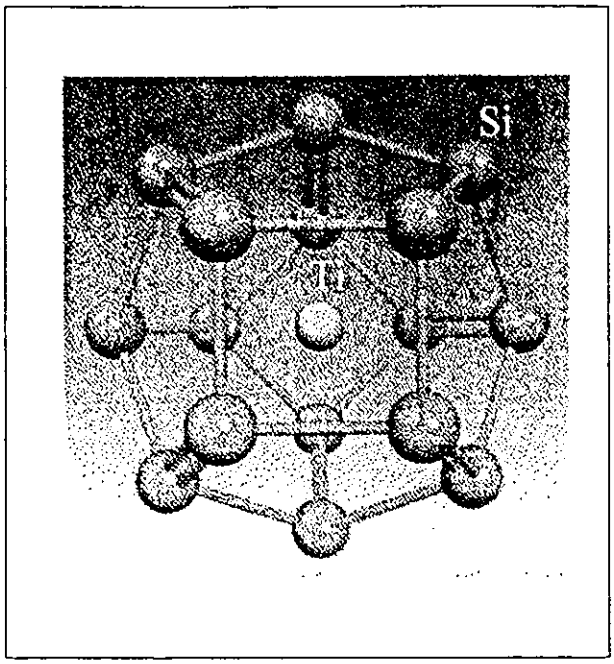
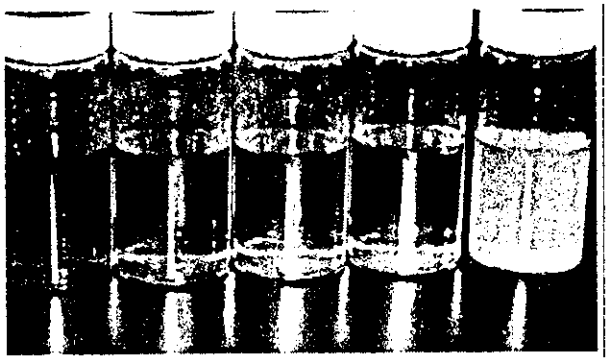


図1　金属内包シリコンフラーレンの一例

ている。以下に述べるナノクラスターはそれぞれが単体として蛍光マーカーとしての機能を有するものがあり，これらは表面の修飾により分子キャリアーとして drug derivery system(DDS)としても利用可能である。ナノクラスターはDDSなどの治療のみならず，分子マーカー，造影剤，センチネルリンパ節マーカーなど診断への利用も期待される。

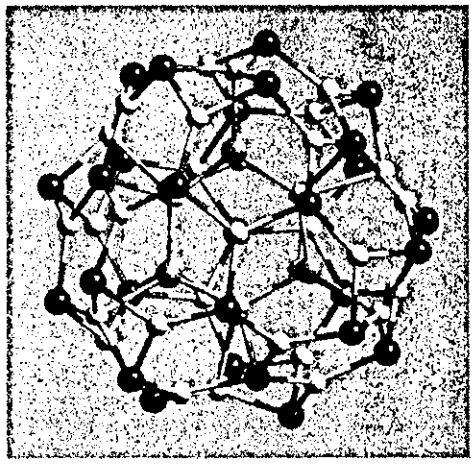
1）カーボンフラーレン，カーボンナノチューブ

すでに広く知られているが，カーボンクラスターの1種である，カーボンフラーレンネットワークのとる構造としては，これまでC60，C70のサッカーボール構造（直径およそ1nm）と，ネットワークが円錐・円筒状（直径数nm）になった構造の2種類が知られてい



3nm　　1nm

粒径によって蛍光色が異なる



Cd/Seナノクラスター
Ⅱ-Ⅵ族半導体(直径1.5nm),
溶液法で作製

図2　Cd/Seナノクラスターと粒経調節による色調の変化

て，C60，C70は1985年にKroto・Smalley，カーボンナノチューブは1991年にIijimaらによって発見された。

Krotoらはフラーレン発見時に様々な原子の内包化により多様な応用が期待できること，実際に酸素原子やランタン原子が内包できることについて述べているが，その後，ベリリウム等金属原子を内包できることが証明された。そしてこの結果を基に新たな応用を視野に入れた研究が展開されつつある。現在のところ，ボーリングボールやエンジンオイルの添加剤などへの利用にとどまっているが，近年表面の修飾による親水化も実現し，DDSのキャリアー等，応用の拡大が期待される。

カーボンナノチューブは単層もしくは渦巻状の角型のフラーレンネットワークである。現在，原子間力顕微鏡の先端チップに利用され，その性能を飛躍的に向上させた。原子間力顕微鏡の分解能は数nmに達している。カーボンナノチューブも今後，もっとも細い導電ケーブルとしてナノエレクトロニクスを始めとした医療応用が期待されるナノクラスターの一つである。

2）シリコンナノクラスター

従来，シリコンには，十量体以上で対称性のよいクラスターは存在しないとされてきた。2001年に川添らの第一原理に基づくスーパーコンピューターを用いた計算によって，金属原子を内包させると，フラーレン型の極めて対称性のよいクラスターが精製できることがKumar，Kawazoeらによって理論的に初めて示された（図1）。

そして2002年には早くもその存在が実験的にOhara，Nakajimaらによって実証された。カーボンフラーレン同様，安定なケージ構造を取っていることから安定と考えられ，蛍光マーカーとしての性質も持つことから今後，幅広い応用が期待される。

3）量子ドット

カドミウム（Cd）とセレナイド（Se）等，Ⅳ族とⅤ族の元素の組み合わせによるこの半導体クラスター（直径2-10nm）は，従来の蛍光色素にない3つの優れた蛍光特性をもつ。

①従来の蛍光色素が特定波長の光を吸収して励起され，特定波長の蛍光を放出するのに比べ，QDはある一定波長以上の光エネルギーはすべて吸収して励起状態になり，基底状態に戻る時は単一波長の蛍光のみ発する。そのため励起効率が極めて高く，従来の蛍光色素の20-30倍に達する。

②材料が無機物であるため有機系の蛍光色素に比べ，極めて高い耐光性を有する。従って繰り返しの励起光照射に耐える。

③同じ材料でも粒径を変えるだけで蛍光波長を自在に変化させることが可能である（図2）。従って同一材料で蛍光波長の異なる粒子を作ることができる。

既にQuantum Dot社からQuantum Dot (QD)が昨年から市販され，さらにさまざまなタイプのQDが各国で開発されている。新たな蛍光マーカーとして様々な角度からの応用が期待されている。

4）オリゴデンドリマー

これは有機デンドリマーと呼ばれ、多数の枝が球から突き出た，いがぐり状を呈するその特異な構造から医療応用，特にDDSの担体としての利用が期待される物質の一つであ

る。サイズはタンパク質とほぼ同等である。特色としては外界との接触面積が非常に大きく，デンドリマーの枝々の間には莫大な空間が存在するため，その間に薬剤を内包させれば，病変部までの薬剤担体として利用可能となる。

5）ペプチドナノキャリアー

キトサンのデオキシコール酸添加による疏水化・ミセルの形成，亜硝酸ナトリウム添加による凝集・解離を利用してサイズ・形状をコントロールできる（径およそ130-300nm）ことからペプチド，薬物，遺伝子などの担体としての利用が期待される。

2. まとめ

ナノテクノロジーは現在急速に発展しつつあるが，特に医療への応用に際しては体内動態等，安全性の確保が必須である。

しかしこのような問題も乗り越え，今後さらに大きく進歩し，ゲノム科学がそうであるように，ナノテクノロジー科学を治療に生かす手段として医学における主要な研究分野となり，かつ大きな産業分野として成長すると考えられる。

〈BIO Information〉

文部科学省ナノテクノロジー総合支援プロジェクト
第2回分野横断スクール「ナノバイオスクール」プログラム

独立行政法人物質・材料研究機構は第2回分野横断スクール「ナノバイオスクール」を開催します。

主催: 独立行政法人物質・材料研究機構　ナノテクノロジー総合支援プロジェクトセンター
開催日程: 平成17年1月17日(月)10:00開講、18日(火)17:00終了
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第1日 1月17日(月):生命の仕組みと運動に学ぶナノテクノロジー
1) 開会の挨拶　文部科学省研究振興局 基礎基盤研究課
2) 開講の挨拶　大阪大学 教授 柳田 敏雄
3) 「生命の仕組みを一分子ごとに追跡する」　大阪大学 教授 柳田 敏雄
4) 「超分子ナノマシンの自己構築とスイッチ機構」　大阪大学 教授 難波 啓一
5) 「回転分子モーター」　東京大学 助教授 野地 博行

第2日 1月18日(火):生命における発生機構と情報伝達に学ぶ
6) 「脳の分節構造にもとづくニューロンの機能分化と機能結合」　大阪大学 助教授 小田 洋一
7) 「生命におけるパターン形成のしくみ」　名古屋大学 教授 近藤 滋
8) 「ゲノムバイオロジーとナノバイオテクノロジーの融合」　かずさDNA研究所部長 小原 収
9) Closing Remarks　東京大学 教授 榊 裕之

応募方法:当センターのフォームよりお申込みください。
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岡村 茂 (電話 03-5404-3286)、鈴木 敦 (電話 03-5404-3282)
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Rapid double 8-nm steps by a kinesin mutant

Hideo Higuchi[1], Christian Eric Bronner[2], Hee-Won Park[3] and Sharyn A Endow[2,*]

[1]Department of Metallurgy and Center for Interdisciplinary Research, Tohoku University, Sendai, Japan, [2]Department of Cell Biology, Duke University Medical Center, Durham, NC, USA and [3]Department of Structural Biology, St Jude Children's Research Hospital, Memphis, TN, USA

The mechanism by which conventional kinesin walks along microtubules is poorly understood, but may involve alternate binding to the microtubule and hydrolysis of ATP by the two heads. Here we report a single amino-acid change that affects stepping by the motor. Under low force or low ATP concentration, the motor moves by successive 8-nm steps in single-motor laser-trap assays, indicating that the mutation does not alter the basic mechanism of kinesin walking. Remarkably, under high force, the mutant motor takes successive 16-nm displacements that can be resolved into rapid double 8-nm steps with a short dwell between steps, followed by a longer dwell. The alternating short and long dwells under high force demonstrate that the motor stepping mechanism is inherently asymmetric, revealing an asymmetric phase in the kinesin walking cycle. Our findings support an asymmetric two-headed walking model for kinesin, with cooperative interactions between the two heads. The sensitivity of the 16-nm displacements to nucleotide and load raises the possibility that ADP release is a force-producing event of the kinesin cycle.

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Introduction

The kinesin motor proteins bind to ATP and microtubules, and use the energy of nucleotide hydrolysis to move along the microtubule. The first discovered or conventional kinesin is a highly processive motor that takes more than a hundred steps each time it binds to a microtubule (Howard *et al*, 1989; Svoboda *et al*, 1993; Hackney, 1995). Steps by conventional kinesin along a microtubule are tightly coupled to ATP hydrolysis—the motor takes a single 8-nm step for each ATP it hydrolyzes (Schnitzer and Block, 1997; Coy *et al*, 1999).

The mechanism by which kinesin walks along a microtubule is not well understood, although several models have been postulated. One model involves a hand-over-hand stepping mechanism in which the two heads of the motor alternate in binding to the microtubule and hydrolyzing ATP, each head in turn taking a step to advance the motor towards the microtubule plus end (Howard, 2001; Schief and Howard, 2001). This model is supported by the finding that processivity of kinesin requires two heads (Hancock and Howard, 1998). It accounts for the observed cooperativity of binding to nucleotide and microtubules by the two heads of kinesin—the finding that only one head of the dimeric motor binds to a microtubule and releases ADP in the absence of ATP, and that ATP hydrolysis by the bound head is required for the other head to bind to the microtubule (Hackney, 1994)—by postulating that both heads participate in ATP hydrolysis in an alternating manner. If both heads hydrolyze ATP, the tight coupling between ATP hydrolysis and steps by the motor requires that both heads be involved in producing force and taking steps along the microtubule.

A hand-over-hand model could involve either a symmetric mechanism in which the rear head always steps to the same side of the forward head to take the next step along the microtubule (Howard, 1996), or an asymmetric mechanism in which the rear head steps to either side of the forward head (Hirose *et al*, 2000; Hoenger *et al*, 2000; Schliwa, 2003). The rationale underlying the proposal of a symmetric hand-over-hand mechanism is that the two heads of the dimeric motor are functionally equivalent and should undergo the same movements and conformational changes during the nucleotide hydrolysis cycle (Howard, 1996). The only available crystal structure of dimeric kinesin shows a rotational symmetry of the two heads around the axis of the coiled-coil stalk that causes the microtubule-binding regions of the heads to be on opposite sides of the motor (Kozielski *et al*, 1997). Thus, a symmetric hand-over-hand model would require that the motor rotate ~180° each time an unbound head binds to the microtubule to take a step. This should produce rotations during processive movement that are detectable experimentally.

Failure to observe the rotational movement predicted by a hand-over-hand mechanism has led to the proposal of an 'inchworm' model in which only one head binds to the microtubule and hydrolyzes ATP, dragging the second head along (Hua *et al*, 2002). This model is consistent with the failure to observe 180° rotations of microtubules bound to single kinesin motors, but does not account for the cooperativity of nucleotide and microtubule binding by the two heads of the motor (Hackney, 1994), which requires that both heads of the motor hydrolyze ATP and produce force. Further, the experimental results do not compel an inchworm model, as they could also be accounted for by an asymmetric hand-over-hand model in which conformational changes of the neck linker or another structural element, together with stepping by the rear head to either side of the forward head in successive steps, produce net rotations too small to be detected in the previous experiments (Hua *et al*, 2002).

Here we report a single amino-acid change of conventional kinesin that affects stepping by the motor. The mutant motor

*Corresponding author. Department of Cell Biology, Duke University Medical Center, 438 Jones Building, Research Drive, Durham, NC 27710, USA. Tel.: +1 919 684 4311; Fax: +1 919 684 8090;
E-mail: endow@duke.edu

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moves processively along the microtubule by taking successive 8-nm steps, like wild-type kinesin, but under high force the motor moves by 16-nm displacements instead of 8-nm steps. Analysis of the 16-nm displacements reveals that the motor stepping mechanism is asymmetric. The asymmetry is not stochastic, but recurs in a regular pattern that compels an asymmetric mechanism for kinesin walking. The load sensitivity of the 16-nm displacements identifies a potential force-producing event of the kinesin cycle.

Results

Mutant design

The kinesin-T94S mutant was designed to make the highly conserved nucleotide-binding motif or P-loop of *Drosophila* kinesin heavy chain, GQTSSGKT, resemble more closely the P-loop of the myosins, GESGAGKT, in amino-acid sequence. The change of T94 to S causes only a small predicted structural change in the motor (Figure 1); however, T94 interacts with the β-phosphate of ADP in the kinesin motors and may help stabilize ADP binding. The point mutation was thus expected to open the nucleotide-binding cleft and permit more rapid nucleotide binding or release by the motor. The mutant motor was expressed in bacteria as a fusion to a biotin-binding protein and tested for ADP release in biochemical assays and velocity of movement along microtubules in *in vitro* motility assays.

ADP release assays

Single-turnover mant-ADP release experiments using FPLC-purified mutant or wild-type motor protein without microtubules showed that, upon addition of 500 μM Mg·ATP, the kinesin-T94S mutant releases ADP ~3.6-fold faster ($0.0128 \pm 0.0013\,s^{-1}$, $n = 11$) than wild-type kinesin ($0.00353 \pm 0.00017\,s^{-1}$, $n = 12$) (Table I). When no nucleotide was added, the mutant motor released ADP at the same rate ($0.00319 \pm 0.00079\,s^{-1}$, $n = 4$) as the wild-type motor with added Mg·ATP, indicating that release of ADP by the kinesin-T94S motor is not dependent on binding of ATP or microtubules. The wild-type motor without added nucleotide released ADP more slowly ($0.00154 \pm 0.00080\,s^{-1}$, $n = 3$), with poorer curve fits (linear correlation coefficient, $R > 0.75$) than the kinesin-T94S mutant ($R > 0.95$).

Single-turnover assays with microtubules were performed by mixing 0.2 μM kinesin-T94S or wild-type kinesin with 1 μM microtubules and monitoring the release of mant-ADP in a fluorometer. Results of these assays showed an acceleration of ADP release by microtubules (9- to 10-fold), which was comparable for the mutant and wild type. The dissociation rate for the kinesin-T94S motor ($0.0287 \pm 0.0187\,s^{-1}$, $n = 9$) was higher than that of wild-type kinesin ($0.0162 \pm 0.0062\,s^{-1}$, $n = 8$) (Table I), but the overall values did not differ significantly due to the variability from assay to assay. Attempts to measure the k_{cat} of the mutant and wild-type motors in ATPase assays with microtubules gave variable values that were lower than normal for wild type and somewhat higher for the mutant. The high variability in mant-ADP release rates in the presence of microtubules and microtubule-stimulated k_{cat} values may be due to the effects of the BIO fusion protein on motor–microtubule interactions when the motor is not bound to a glass surface or bead, as motor velocities in gliding and laser-trap assays were in the normal range for wild-type kinesin (see below). The k_{cat} values with microtubules are not reported here to avoid misinterpretation of the mutant effects. Detailed studies of nonfusion mutant and wild-type motors in the presence of

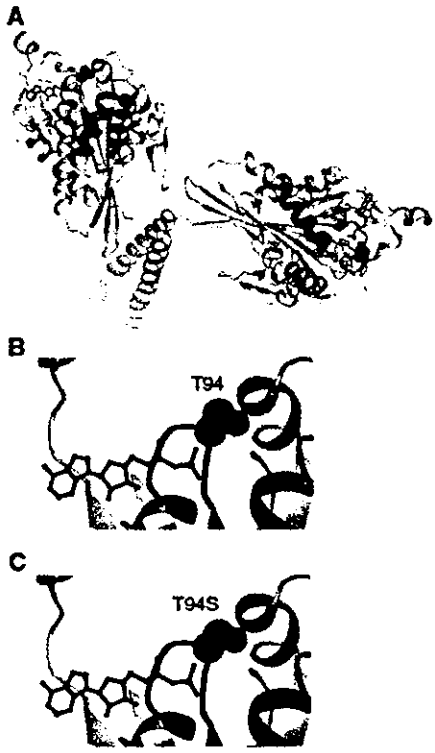

Figure 1 Kinesin-T94S mutant. (**A**) Structure of wild-type kinesin. The atomic structure of the dimeric motor is shown as a ribbon diagram (rat kinesin heavy chain, PDB 3KIN) (Kozielski *et al*, 1997). The conserved T94 in the nucleotide-binding P-loop is space-filled (purple) and the P-loop (GQTSSGKT) is green. (**B**) Close-up of the active site with the residue corresponding to *Drosophila* kinesin T94 in purple. (**C**) The active site with S94 (cyan) modeled into the wild-type structure in place of T94. ADP, wire diagram; helices α4 and α5, black.

Table I Kinetic properties of the kinesin-T94S and wild-type kinesin motors

	Kinesin-T94S	Wild-type kinesin
k_d, ADP release–ATP	$0.00319 \pm 0.00079\,s^{-1}$, $n = 4$, $R > 0.95$	$0.00154 \pm 0.00080\,s^{-1}$, $n = 3$, $R > 0.75$
k_d, ADP release + ATP	$0.0128 \pm 0.0013\,s^{-1}$, $n = 11$, $R \geq 0.90$	$0.00353 \pm 0.00017\,s^{-1}$, $n = 12$, $R > 0.93$
k_d, ADP release + MTs	$0.0287 \pm 0.0187\,s^{-1}$, $n = 9$, $R \geq 0.73$	$0.0162 \pm 0.0062\,s^{-1}$, $n = 8$, $R \geq 0.65$
Velocity, MT gliding	255 ± 13 nm/s, $n = 20$	853 ± 25 nm/s, $n = 19$

Values are the mean ± s.e.m.; *R*, linear correlation coefficient for the curve fit (values closer to 1.00 indicate a better fit); MT, microtubules.

microtubules will be required to determine the effects of the T94S mutation on motor binding to nucleotide in the presence of microtubules. The altered rate of ADP release by the kinesin-T94S mutant in the absence of microtubules and the initial data presented here for assays with microtubules raise the possibility that ADP release in the presence of microtubules is also altered.

Gliding assays

Microtubule gliding assays (Song *et al*, 1997) were performed with lysates of the kinesin-T94S or wild-type motor. The assays showed good binding to microtubules by the motors attached to the coverslip, but a slower gliding velocity by ~3.3-fold for the mutant (255±13 nm/s, $n=20$) compared to wild type (853±25 nm/s, $n=19$) (Table I).

Laser-trap assays

The increased ADP release rate in the absence of microtubules, but decreased velocity in gliding assays, suggested that stepping of the kinesin-T94S mutant along microtubules might be altered. Traces of single kinesin-T94S motors in laser-trap assays showed slow movement compared to wild type (Figure 2A and B). The velocity of the mutant at low load (~1 pN) was 250 nm/s, which was about one-third the velocity of wild type, 760 nm/s, consistent with the gliding assays. The stall force of ~8 pN for the mutant was similar to

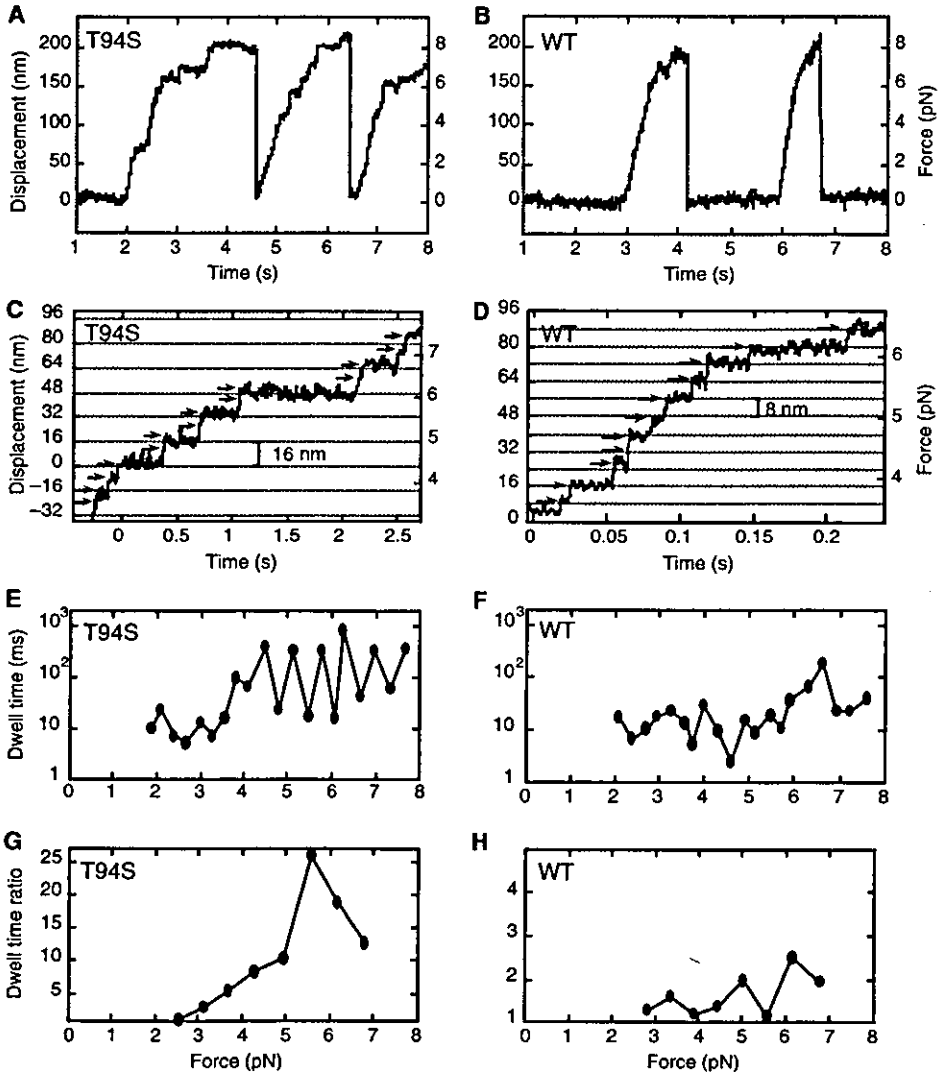

Figure 2 Single-molecule laser-trap assays of kinesin-T94S and wild-type kinesin. (**A, B**) The traces show processive movement by single kinesin-T94S (magenta) and wild-type kinesin (green) motors. (**C, D**) Single kinesin-T94S motors showed successive 8-nm steps at forces < ~4 pN, but exhibited successive 16-nm displacements, consisting of rapid double 8-nm steps, at forces > ~4 pN. Single wild-type kinesin motors under the same conditions showed sequential 8-nm steps. (**E, F**) A plot of dwell time versus force shows alternating short and long dwells following sequential 8-nm steps by the kinesin-T94S mutant. The wild type shows variable dwells following sequential 8-nm steps. (**G, H**) The ratios of the long dwell times divided by the short dwell times at different forces, calculated by averaging overlapping sets of three successive odd and even steps (see Materials and methods) corresponding to the traces in (C, D). The dwell time ratios or limp factors L of the mutant are significantly greater than those of wild type at forces > ~4 pN. Note the difference in Y-axis scales in (G, H).

wild type. The mutant was processive with a step size of 8 nm at low load (< ~4 pN) (Figure 2C). Remarkably, at high load, the mutant showed frequent 16-nm displacements, which could be resolved into two rapid 8-nm steps with a short dwell between steps followed by a longer dwell, with the short and long dwells alternating between successive 8-nm steps (Figure 2C and E). The wild-type motor showed 8-nm steps, as reported by others (Svoboda *et al*, 1993; Nishiyama *et al*, 2002), with variable dwells between steps (Figure 2D and F).

The dwell times between steps by the mutant and wild-type motors were analyzed statistically. Steps in traces were assigned as odd or even, and the dwell times for three sequential odd steps and three sequential even steps were averaged (total = 6 steps). The longer mean dwell time was divided by the shorter one to obtain the ratio. Two steps were then slipped from the first step, the next dwell time ratio was calculated, and this process was reiterated for the length of the run. Analysis of the traces for the mutant and wild type in Figure 2C and D is shown in Figure 2G and H.

In all, 31 traces of the mutant and 20 traces of wild type were analyzed in this way without selecting for 8- or 16-nm displacements (Figure 3). The dwell time ratio, referred to as the limp factor *L* (Asbury *et al*, 2003), increased sharply for the mutant from 2.99 to 6.35 with 2.4–6.9 pN force (Figure 3A). By contrast, the limp factor of wild-type kinesin increased slowly from 1.88 to 2.57 with 2.4–6.7 pN force, remaining at a low ratio of <3 (Figure 3A).

The average dwell time of the kinesin-T94S mutant was ~2.5 times longer than that of wild type (mutant = 37 ms and wild type = 14.6 ms at 2.4 pN; mutant = 159 ms and wild type = 61 ms at 6.7–6.9 pN) (Figure 3B and C). For the mutant, the long dwell times increased sharply with force, whereas the short dwell times increased more gradually. The short dwell times of the mutant were longer than the average dwell times of wild type at low force, but they were shorter at high force.

The 16-nm displacements, characterized by short dwell times and high *L* values, appeared clearly at high force (≥4 pN), where they were observed frequently for single kinesin-T94S motors (*L*>5, 32% of dwell time ratios, total = 149, compared to 22% at <4 pN, total = 74). The 16-nm displacements were observed infrequently for wild type (2% of dwell time ratios, total = 148).

At low force, single kinesin-T94S motors showed 8-nm steps at higher frequency than at high force, rather than 16-nm displacements. The appearance of the 16-nm displacements at high force indicates the existence of a phase in the kinesin-T94S walking cycle that can be accelerated by force, resulting in the rapid double 8-nm steps. The 8-nm steps by the mutant were also observed at low ATP concentration (10 μM) (*L* = 2.37 at 2.3–6.2 pN force). The low frequency of 16-nm displacements by the mutant at low ATP concentration (*L*>5, 5% of dwell times, total = 99) is probably due to the time required to release ADP and bind ATP. The rate-limiting step of the kinesin cycle is thought to be the release of ADP (Hackney, 1988). But because the dwell time for the mutant at 10 μM ATP was ~3 times that at 1 mM ATP, the rate-limiting step at low ATP concentration is likely to be ATP binding rather than ADP release, making the dwell time between steps by the mutant close to that of wild type.

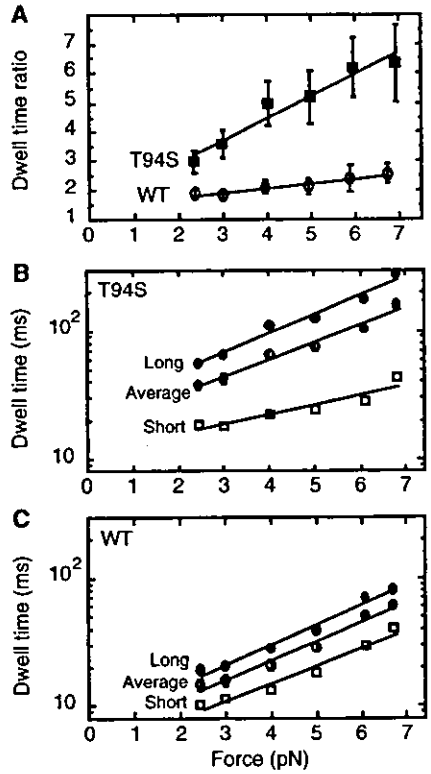

Figure 3 Dwell times versus force for steps by kinesin-T94S and wild-type kinesin. (**A**) The dwell time ratio or limp factor for 8-nm steps by the kinesin-T94S mutant (magenta) or wild-type kinesin (green) increases linearly with force, but the increase is significantly greater for the mutant than wild type. (**B**) The increase in long dwell times of the mutant with increasing force is paralleled by the increase in the average dwell times, while the short dwell times increase more slowly. (**C**) The long and short dwell times of wild-type increase in parallel with one another and with the average dwell times with increasing force. The error bars show the standard error of the mean (s.e.m.) for the dwell time ratios and average dwell times.

Discussion

A kinesin stepping mutant

We report here a new mutant of *Drosophila* kinesin heavy chain that was rationally designed to make the nucleotide-binding P-loop resemble more closely that of the myosins. The mutant motor has a change of T94S in the P-loop, which was expected to open the nucleotide-binding cleft and increase the rate of nucleotide binding or release by the motor. This relatively minor structural change causes the motor to release ADP ~3.6-fold faster than wild type in the absence of microtubules and to translocate microtubules in gliding assays with a velocity ~3.3-fold slower than wild type. In single-molecule laser-trap assays, the mutant motor shows frequent 16-nm displacements under high force, which are resolvable into rapid double 8-nm steps, consisting of alternating slow and fast steps. At low force or low ATP concentration, successive 8-nm steps were observed instead of 16-nm displacements. The wild-type motor showed infrequent 16-nm displacements due to rapid double 8-nm steps.

The frequency of rapid double 8-nm steps is thus enhanced in the mutant compared to wild type.

The 16-nm displacements arise by alternating fast and slow dwell times, causing alternate steps to be fast and slow. The structural basis of a slow step by the kinesin-T94S mutant that alternates with a fast step could be due to an inherent asymmetry of stepping to the right or left of the forward head caused by the handedness of the twist of the coiled coil. Stepping to the right might be constrained by the twist of the coiled coil, whereas stepping to the left might not be, and might be accelerated by interactions of the rear head with the forward head.

The successive 16-nm displacements in the kinesin-T94S traces with alternating short and long dwell times indicate that a step in the kinesin walking cycle is asymmetric, as predicted by asymmetric two-headed walking models, but not by symmetric ones such as hand-over-hand models in which the rear head always steps to the same side of the forward head (Howard, 1996), or inchworm models, in which only one head steps, dragging the other head along (Hua *et al*, 2002). Our results favor an asymmetric walking model in which the two heads of kinesin alternate in binding to the microtubule and hydrolyzing ATP.

Stepping by heterodimeric kinesin

Recently, others have reported 16-nm displacements by a heterodimeric kinesin protein consisting of kinetically different mutant and wild-type motor subunits (Kaseda *et al*, 2003), providing evidence that the two heads of the heterodimeric motor alternate in stepping. The interactions of the two heads of a heterodimeric motor with one another could differ from that of the native motor, however, due to the mutation in one head or due to the different proteins to which they were fused. The results we report here differ from those of Kaseda *et al* (2003) in that we observe asymmetry of movement for a homodimeric kinesin motor. This substantiates these previous findings by showing that the asymmetric steps of the heterodimer must have been due to an inherent asymmetry of stepping by the motor and were not dependent on the differences in the motor subunits.

Limping by wild-type kinesin motors

Our findings differ from those reported recently by others (Asbury *et al*, 2003) for wild-type *Drosophila* and native squid kinesin. These workers found evidence for alternating slow and fast steps under high force (4 pN) for truncated *Drosophila* kinesin proteins, whereas we did not observe frequent 16-nm displacements for our truncated wild-type kinesin, which was expressed as a fusion to a 106-residue BIO protein with two linker residues. The basis of these conflicting results could be a difference in the kinesin preparations used in the assays, a dependence of limping on the total length of the proteins analyzed, such that shorter proteins show a greater tendency to limp because they interact asymmetrically with the surface of the bead rather than the microtubule, or the conditions of the laser-trap assays. In the present work, we used a kinesin motor fused to a BIO domain to prevent interactions of the motor with the bead.

The velocities reported by Asbury *et al* (2003) both for their truncated and native kinesin proteins were unusually slow, ~65–165 nm/s (calculated from the mean fast and slow dwell times in their Figure 3C). These velocities are significantly slower than the velocities of 300 and ~600 nm/s for squid kinesin at 4 pN reported previously by the same laboratory (Visscher *et al*, 1999; Block *et al*, 2003), and the velocity of 390 nm/s at 4.0 pN of our wild-type kinesin, DmK447-BIO. These unusually slow velocities could arise if the force-clamp assays were performed under higher actual force than the reported value of 4 pN, or the kinesin proteins used in the assays were heterogeneous, comprising some motors in which one head was slower than the other due to partial inactivation during purification. The slow velocities of the truncated and native kinesin motors reported by Asbury *et al* (2003) need to be accounted for and correlated with the limping behavior of the motors before attempting to reconcile our data with theirs.

Kinesin-T94S and stepping models

The results we report here are important in that they show that a kinesin mutant with a single amino-acid change in the nucleotide-binding P-loop, under high force, shows a high frequency of rapid double 8-nm steps, resulting in 16-nm displacements. Under low force or low ATP concentrations, the mutant takes 8-nm steps, like wild-type kinesin. This suggests that the mutation enhances a step in the wild-type kinesin stepping cycle that is sensitive to load and the nucleotide state of the motor. Load-sensitive conformational changes have not previously been identified for kinesin and are believed to correspond to the force-producing steps of the hydrolysis cycle. The finding that the mutant motor is altered in ADP release tentatively identifies ADP release as a force-generating event of the kinesin cycle, although it is still possible that a different step of the hydrolysis cycle is affected. Further biochemical studies will be required to determine the step in the cycle that is altered in the mutant and how it affects the motor stepping mechanism. The observation of rapid double 8-nm steps under high load with alternating long and short dwells implies that a phase of the stepping cycle is affected by the mutant that involves the alternative regulation or interactions of the two heads.

The disappearance of the 16-nm displacements at low force or low ATP concentration indicate that another rate-limiting step, probably ATP binding, suppresses the rapid double 8-nm steps by interposing a longer dwell between the steps, resolving them into two steps. The ability of the kinesin-T94S motor to take successive 8-nm steps demonstrates that the basic mechanism of walking by the kinesin motor is unaltered. The appearance of the rapid double 8-nm steps or 16-nm displacements at high force and their infrequent appearance in wild-type traces indicates that the mutant enhances a step in the walking cycle that is inherently asymmetric, revealing an asymmetric walking mechanism for kinesin.

Materials and methods

Plasmids

Plasmids to express the kinesin-T94S mutant or corresponding wild-type *Drosophila* kinesin heavy chain (KHC) motor fused to a biotin-binding protein were constructed by inserting DNA fragments synthesized by the polymerase chain reaction (PCR) into *pMW172/KHC* (Song and Endow, 1996). The plasmids encode M-G-S+KHC residues E4-Q447, linked by G-S to 106 residues of the *Propionibacterium shermanii* biotin-binding transcarboxylase (BIO)

(Pinpoint Xa, Promega). PCR-synthesized regions were confirmed by DNA sequence analysis.

Protein expression and purification
The kinesin-T94S and corresponding wild-type kinesin proteins fused to BIO were expressed in bacteria using the T7 RNA polymerase system (Studier *et al*, 1990). The fusion proteins were 123.4 kDa dimers of a 567-residue subunit. The kinesin region of the fusion protein corresponds to the conserved motor domain (E4-F326), the neck linker (G327-T344) and 103 residues of the coiled coil (A345-Q447). Proteins were purified for biochemical assays by chromatography on P11 phosphocellulose, followed by MonoQ and/or Superose 12 FPLC. The Superose 12 chromatography step was necessary to remove high-molecular-weight kinesin aggregates. The purified proteins were diluted extensively for use in laser-trap assays.

Motility assays
Microtubule gliding assays on motor-coated coverslips were performed as described (Song *et al*, 1997), using lysates of the kinesin-T94S or wild-type motors fused to BIO.

ADP release assays
Single-turnover mant-ADP release experiments were performed using FPLC-purified kinesin-T94S or wild-type protein fused to BIO by incubating 2 μM mant-ATP [2′(3′)-*O*-(*N*-methyl-anthraniloyl)-adenine 5′-triphosphate] with 0.5 μM motor for ⩾2 h on ice in the dark. Samples were warmed to room temperature for 5–10 min and fluorescence (excitation, 356 nm; emission, 446 nm) was recorded in a SPEX FluoroMax or SLM Aminco 8100 spectrofluorometer before and after addition of 500 μM ATP. Mant-ADP release assays in the presence of microtubules were performed by mixing 0.2 μM motor + mant-ATP after incubation on ice with 1 μM GTP-depleted microtubules in a cuvette and recording fluorescence before and after addition of the microtubules. Fluorescence was plotted versus time and data points were fit to a single exponential curve,

$$y = m_3 + m_1 e^{(-m_2 m_0)}$$

where y = fluorescence (cps), m_0 = time (s) and $y = m_3 + m_1$ at $t = 0$, using KaleidaGraph v 3.08d. Dissociation rate constants ($k_d = m_2$) were obtained from the curve fits. In some experiments, samples were spin-dialyzed to remove excess mant-ATP after incubation on ice and before recording fluorescence; rates were the same as assays in which mant-ATP was not depleted. Data for the experiments were therefore combined.

Laser-trap assays
Laser-trap assays were performed using highly diluted purified kinesin-T94S or wild-type motors expressed as fusions to BIO and attached to 0.2 μm avidin-coated beads in buffer + 200 mM NaCl + 1 mM ATP (Endow and Higuchi, 2000; Nishiyama *et al*, 2002). The data were derived from single motors, based on the statistics of binding of the beads to microtubules (Svoboda and Block, 1994; Endow and Higuchi, 2000). Assays performed at low ATP concentrations contained 10 μM ATP. Beads were trapped at a stiffness of 0.04 pN/nm and monitored for binding and movement on microtubules. Data were taken without a low-pass filter, then filtered through 50 Hz (Figure 2A and B) and 200 Hz (Figure 2C and D) low-pass filters for analysis.

Statistical analysis
Dwell times between steps of the mutant and wild type were analyzed statistically, as described by others (Asbury *et al*, 2003). Steps in a given trace were assigned as odd or even, and dwell times of three sequential odd steps and three sequential even steps were averaged. The longer mean dwell time was divided by the shorter one to obtain the dwell time ratio. Two steps were slipped along the trace from the first step, the next dwell time ratio was calculated from the next overlapping six steps, and this process was reiterated for the length of the run. The theoretical ratio for an exponential distribution of random dwell times generated by computer simulation was calculated to be 2.23 for analysis of overlapping sets of six sequential steps and 1.84 for 10 sequential steps. These values were close to the value of 1.79 obtained by analyzing dwell times from computer-simulated stepping records (Asbury *et al*, 2003).

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Motility of myosin V regulated by the dissociation of single calmodulin

HoaAnh Nguyen[1–3] & Hideo Higuchi[1,3]

Myosin V is a calmodulin-binding motor protein. The dissociation of single calmodulin molecules from individual myosin V molecules at 1 μM Ca^{2+} correlates with a reduction in sliding velocity in an *in vitro* motility assay. The dissociation of two calmodulin molecules at 5 μM Ca^{2+} correlates with a detachment of actin filaments from myosin V. To mimic the regulation of myosin V motility by Ca^{2+} in a cell, caged Ca^{2+} coupled with a UV flash system was used to produce Ca^{2+} transients. During the Ca^{2+} transient, myosin V goes through the functional cycle of reduced sliding velocity, actin detachment and reattachment followed by the recovery of the sliding velocity. These results indicate that myosin V motility is regulated by Ca^{2+} through a reduction in actin-binding affinity resulting from the dissociation of single calmodulin molecules.

Calcium regulates various enzyme reactions of cellular processes through direct interaction to modify the function of a protein or through indirect interaction with a Ca^{2+}-sensing protein. Among the Ca^{2+}-sensing proteins expressed in eukaryotic cells, calmodulin (CaM) is a member of the EF-hand family, and has been known to participate in many signaling pathways that affect crucial processes such as cell growth, proliferation and cell movement[1,2].

Myosin V is an unconventional double-headed myosin that transports synaptic and endoplasmic reticulum vesicles in neurons[3–5], pigment granules in melanocytes[6–8], and vacuoles in yeast[9,10]. Myosin V is a processive motor capable of taking 36-nm steps along actin filaments[11–13]. Structurally, myosin V is a homodimer. Each monomer has one N-terminal head domain, one extended neck domain that contains six IQ motifs, and a tail domain containing a coiled coil region attached to a C-terminal globular region[14–16]. The IQ motifs form binding sites for CaM and CaM-related light chains[14,16,17]. Recent studies have investigated whether CaM bound to the IQ motifs can regulate the function of myosin V in response to Ca^{2+} (refs. 16,18–21). Two of those studies have reported that myosin V stops moving but remains bound to actin filaments at high Ca^{2+} concentrations (>1 μM) in *in vitro* motility assays using nitrocellulose coated glass[19,20]. In this study, using an *in vitro* motility assay on uncoated glass slides, we observed the detachment of actin filaments from myosin V at high Ca^{2+} concentrations. This finding suggests that high Ca^{2+} concentrations are a novel way of inactivating the motility of myosin V. Furthermore, upon transient release of Ca^{2+}, myosin V reversibly detaches from and slides along actin filaments in the presence of free CaM. To provide insights into the underlying mechanism of this observation, we used single-molecule imaging to monitor the dissociation of individual CaM molecules from myosin V at various Ca^{2+} concentrations. Based on these observations, we propose a model explaining the regulation of the myosin V motor function resulting from the dissociation of single CaM molecules. This model shows how a motor protein detaches and then reattaches to their moving track. This may also explain how a multimotor-binding vesicle could be switched between different tracks for targeting in a cell.

RESULTS

Effect of Ca^{2+} on myosin V motility

To estimate the effect of Ca^{2+} on myosin V motility, we first developed a suitable glass surface for observing reversible motility in the *in vitro* assay (**Supplementary Methods** online). Using nitrocellulose-coated glass, movement was restored for only 56 ± 16% (mean values ± s.d., $n = 15$) of the actin filaments after removing Ca^{2+} in the solution. Using an uncoated glass surface, 96 ± 3% ($n = 15$) of the actin filaments showed movement. Thus, the remainder of our experiments were done using uncoated glass slides. The irreversible inactivation of myosin V on nitrocellulose-coated glass was most likely due to irreversible binding of myosin V molecules to the nitrocellulose at the hydrophobic site of IQ domains (containing ~40% hydrophobic residues)[15], which is exposed after the dissociation of CaM.

The *in vitro* motility assays for myosin V were done in the presence of various Ca^{2+} concentrations. At pCa 6.0, the number of sliding actin filaments was less than that in the absence of Ca^{2+} (pCa > 7.0). At pCa 5.6, all actin filaments detached from myosin (**Fig. 1a**). To analyze the effect of Ca^{2+} in detail, we quantified the number and velocity of actin filaments (**Fig. 1b,c**). In the absence of Ca^{2+} or at pCa 6.4, the density of sliding actin filaments was ~0.17 filaments μm^{-2}, and most of them (>95%) moved smoothly. However, at pCa 6.0 there was a large reduction in both the density of sliding filaments and velocity of sliding. At pCa 5.8, the velocity of a few actin filaments could still be observed. It was similar to that recorded at pCa 6.0 (~230 nm s^{-1}). Finally, at

[1]Center for Interdisciplinary Research, Tohoku University, Sendai 980-8578, Japan. [2]Laboratory of Applied Microbiology, Department of Molecular and Cell Biology, Division of Life Science, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan. [3]Biomedical Engineering Research Organization, Tohoku University, Sendai 980-8575, Japan. Correspondence should be addressed to H.H. (higuchi@material.tohoku.ac.jp).

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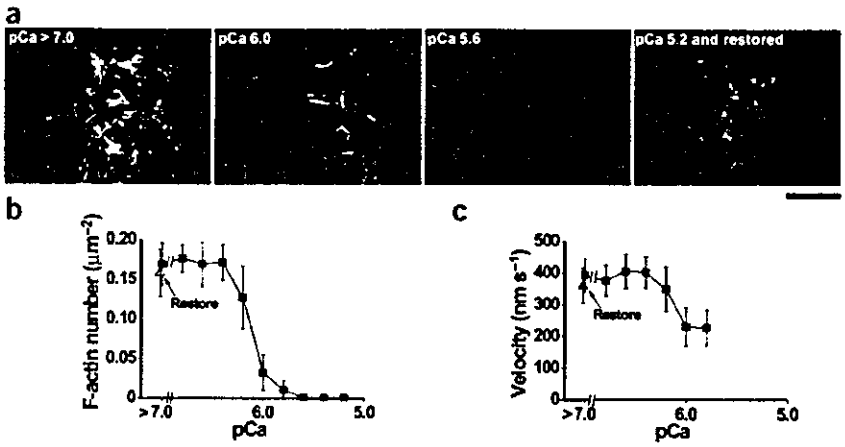

Figure 1 Effects of Ca^{2+} on the *in vitro* motility assay of myosin V. (**a**) *In vitro* motility assays carried out in the absence of Ca^{2+} (pCa > 7.0), and at pCa 6.0 and 5.6. At pCa 5.2, movement was restored by washing out Ca^{2+} in the presence of 10 μM CaM. Bar, 10 μm. (**b**,**c**) The number (**b**) and velocity (**c**) of sliding actin filaments (F-actin) at different pCa values in the *in vitro* motility assay. Data in **b** and **c** are mean values ± s.d. from three independent experiments.

pCa ≤ 5.6, all actin filaments were detached from myosin V, and thus sliding velocity could not be measured. Both the number and the velocity of sliding filaments could be restored to original levels by removing Ca^{2+} from the solution in the presence of 10 μM CaM (**Fig. 1**) but not in the absence of CaM (data not shown).

To determine the reason for the reduction in both the motility and the actin-binding affinity of myosin V, the number of CaM dissociated from myosin V in the presence of Ca^{2+} was estimated using single-molecule analysis. The dissociated CaM was substituted with Cy3-calmodulin (*CaM) and the number and intensity of fluorescent spots of *CaM were observed at single-molecule level (**Fig. 2a** and **Supplementary Methods** online). At pCa values of 6.0 and 5.8 (**Fig. 2b**), at least one CaM dissociated from each myosin V as the number of fluorescent spots (~0.19 spots μm^{-2}) of the substituted *CaM inside the chamber was almost equal to the calculated number of myosin V molecules initially applied to the chamber (binding of 0.025 nM myosin to the total contact area of 47×10^7 μm^2 in a chamber of 6 μl would give a value of ~0.19 molecules μm^{-2}). This number did not change after several washes with the Ca^{2+} solution followed by substitution with *CaM. In the absence of Ca^{2+} (pCa > 7.0) (**Fig. 2b**), only very few fluorescent spots could be detected (~10 spots) indicating that most of myosin V (~95%) was saturated with CaM before treatment with Ca^{2+}.

To quantify the effect of Ca^{2+} on the dissociation of CaM from myosin V, the dissociated CaM was substituted with *CaM and the population of single, double and triple substitutions of *CaM molecules (**Fig. 2c** and **Supplementary Methods** online) on the myosin V were estimated as a function of Ca^{2+} concentration (**Fig. 2d**). At pCa 6.2, ~50% of the myosin V molecules dissociated a single CaM molecule. At pCa 6.0, ~70% of myosin V dissociated a single CaM molecule, and ~30% of the myosin V dissociated two molecules. The dissociation of two CaM from one myosin V reached saturating levels at pCa 5.6. The triple bleaching steps were negligibly small (<5%) at all Ca^{2+} concentrations, whereas four or more bleaching steps were not observed. This suggests that each myosin V could lose a maximum of two CaM. To ensure that two was the maximum number, a sample was treated three times at pCa 5.2 after *CaM substitution and the number of spots having triple bleaching steps was still <5%.

Because it is well known that one CaM has four calcium-binding sites[3], the number of Ca^{2+} that binds to a CaM and triggers the dissociation of that CaM was estimated. The data for the dissociation of CaM from a myosin V in the presence of Ca^{2+} could be well fitted to the Hill equation giving a best fit value of 3.6 (**Fig. 2e**), suggesting that 3–4 Ca^{2+} are required to trigger the dissociation of one CaM.

Ca^{2+}-dependent dissociation rate of CaM

To determine the kinetic effect of Ca^{2+} on the regulation of myosin V motility, the dissociation rate of CaM from myosin V in the presence of Ca^{2+} was estimated by substituting the dissociated CaM with 100 nM *CaM. To answer whether the association rate of 100 nM *CaM to myosin V affected the estimation of dissociation rate or not, the association rate of 100 nM *CaM to myosin V had to be determined first (**Supplementary Methods** online). The time course of *CaM substitu-

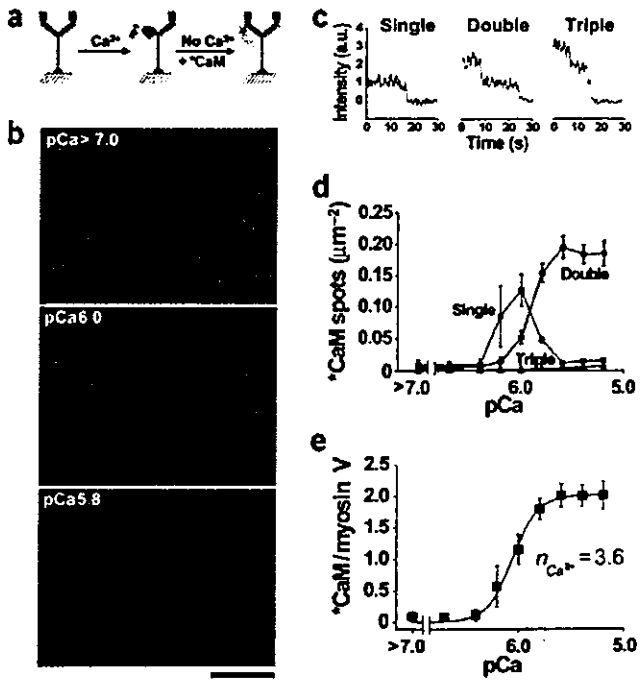

Figure 2 Effect of Ca^{2+} on the dissociation of CaM from myosin V. (**a**) Myosin V was treated with Ca^{2+} at various concentrations. The free Ca^{2+} was washed out and the dissociated CaM was substituted with *CaM. (**b**) Fluorescent spots of the substituted *CaM bound to individual myosin V on the glass surface were observed under TIRFM without Ca^{2+} treatment (pCa > 7.0) or after Ca^{2+} treatments at pCa 6.0 and 5.8. The density of myosin V was ~0.19 molecules μm^{-2} on the observation area (1,000 μm^2). Bar, 10 μm. (**c**) The bleaching steps of fluorescent intensity of single, double and triple *CaM molecules. (**d**) Number of *CaM substituted for CaM that dissociated from individual myosin V at different Ca^{2+} concentrations. (**e**) Dissociation of CaM (replaced with *CaM) from myosin V. Data were collected from D and fitted with the Hill equation[36] to determine the number of Ca^{2+} bound to CaM when CaM dissociates from myosin V. Data in **d** and **e** are mean values ± s.d. from three independent experiments.

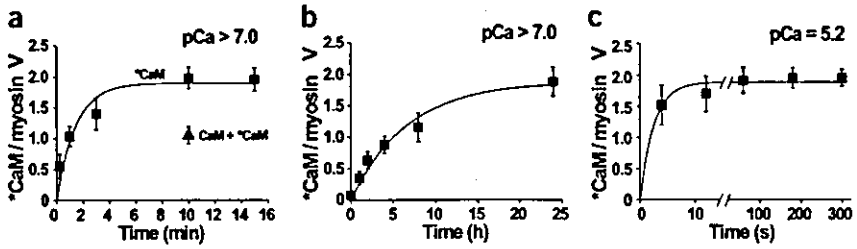

Figure 3 Ca^{2+}-dependent dissociation rate of CaM from myosin V (**Supplementary Methods** online). (**a**) The time course at 100 nM of *CaM substitution on myosin V that had lost a CaM in the absence of Ca^{2+}. Data were fitted with an exponential curve to obtain the association constant of *CaM to myosin V in the absence of Ca^{2+}. (**b**) The time course of *CaM exchange for CaM dissociated from myosin V in the absence of Ca^{2+}. Data were fitted with an exponential curve to obtain the dissociation constant of CaM from myosin V in the absence of Ca^{2+}. (**c**) The time course of CaM dissociated from myosin V at pCa 5.2, which was determined from the substitution of *CaM. Data were fitted with an exponential curve to obtain the dissociation constant of CaM from myosin V at pCa 5.2. Data are mean values ± s.d. from three independent experiments.

tion on myosin V was fitted to an exponential curve (**Fig. 3a**) to determine the pseudo first-order association rate constant ($[*CaM]k_{7.0+}$) at 100 nM *CaM in the absence of Ca^{2+}. To estimate the second-order association rate constant ($k_{7.0+}$), two similar experiments were done at 20 and 50 nM *CaM. The best-fit line on the graph of *CaM concentrations versus first-order association rate constants showed that $k_{7.0+}$ was ~1×10^5 M^{-1} s^{-1} (data not shown). To confirm that labeling with Cy3 does not affect the association rate of *CaM, a 1:1 mixture of nonlabeled CaM and *CaM (100 nM each) was used in a similar experiment as described above. After a 10-min incubation, the number of re-bound *CaM was reduced to ~50% (**Fig. 3a**), indicating that Cy3 labeling had no substantial effect on the association rate of *CaM.

To measure the dissociation rate of CaM from myosin V in the absence of Ca^{2+}, myosin V bound to a glass surface was incubated with 100 nM *CaM for various periods of time up to 24 h (**Supplementary Methods** online). The data were fitted to an exponential curve (**Fig. 3b**) and the dissociation rate constant ($k_{7.0-}$) in the absence of Ca^{2+} was ~0.4×10^{-5} s^{-1}. The dissociation constant (K_d) of CaM from myosin V in the absence of Ca^{2+} was calculated as $k_{7.0-} / k_{7.0+}$, giving a value of ~0.4 nM. To determine the dissociation rate of CaM in the presence of Ca^{2+} (**Supplementary Methods** online), the time course of CaM dissociated from myosin V at pCa 5.2 was fitted to an exponential curve (**Fig. 3c**). The dissociation rate constant ($k_{5.2-}$) at pCa 5.2 was ~0.4 s^{-1}, which is 10^5 times faster than that in the absence of Ca^{2+}.

Regulation of myosin V by caged Ca^{2+}

The conventional *in vitro* motility assay does not show the transient effect of Ca^{2+} on myosin V that normally occurs in cells. To overcome this limitation, the sliding of the filaments was regulated by incorporating an *in vitro* motility assay with caged Ca^{2+} that can generate Ca transients using a UV flash (**Supplementary Methods** online). The duration of the UV flash is 4 s. Before the UV flash, actin filaments moved smoothly on the glass surface (**Fig. 4a**(i)). At the end of the UV flash, most of actin filaments had detached (**Fig. 4a**(ii)), and 40 s after the completion of the UV flash most of them had reattached (**Fig. 4a**(iii)) (**Supplementary Video 1** online).

The time course of the Ca^{2+} concentration was measured using Rhod-2 (ref. 22), a Ca^{2+}-sensitive fluorophore (**Fig. 4b**). Quantification of the number of sliding actin filaments and velocity at which these filaments were moving under various concentrations of CaM show that, before the UV flash, there were ~0.15 actin filaments μm^{-2} sliding at ~400 nm s^{-1} (**Fig. 4c,d**). Within a second after starting the UV flash, actin filaments became detached in the illumination area (**Fig. 4c**). In the absence of free CaM, the number of sliding actin filaments did not increase, even after several minutes (data not shown). In contrast, in the sample containing free CaM, actin filaments were able to gradually reattach and the rate of reattachment was dependent on the free CaM concentration. During the UV flash and before detachment of the actin filaments, the velocity slowed to ~230 nm s^{-1}. When the UV illumination was completed, the velocity was gradually increased and the acceleration was dependent on the free CaM concentration (**Fig. 4d**). The velocity of sliding in the samples could not be measured without adding free CaM, as otherwise the actin filaments did not attach. The number and velocity of sliding actin filaments remained unchanged using UV flash in the absence of caged Ca^{2+} (data not shown). In conclusion, using photolysis of caged Ca^{2+}, we observed the transient regulation of the number of actin filaments and their sliding velocity in the *in vitro* motility assay of myosin V. The movement commenced with detachment and then slowed within a few seconds. Finally, the filaments reattachment and movement recommenced in the presence of free CaM.

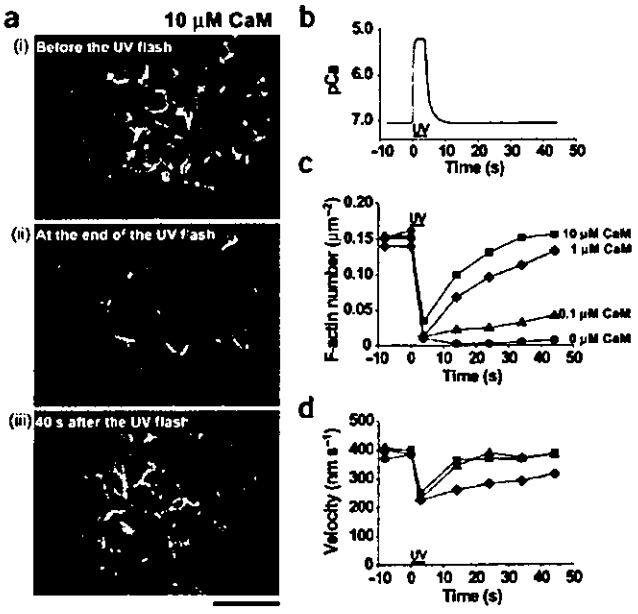

Figure 4 Reversible regulation of myosin V function with transient Ca^{2+} generated by UV photolysis of caged Ca^{2+}. The *in vitro* motility assay was carried out in the presence of transient Ca^{2+} concentrations, which were generated locally by UV photolysis of caged Ca^{2+} within a time frame of 4 s. (**a**) Experiments in the presence of 10 μM CaM at the moment just before (i), at the end of the flash (ii), and 40 s after the 4-s UV photolysis of caged Ca^{2+} (iii). Bar, 10 μm; circle, UV illumination area (**Supplementary Video 1** online). (**b**) The transient Ca^{2+} concentration generated by a 4-s flash of UV photolysis of caged Ca^{2+} was measured using Rhod-2 as the signal. (**c,d**) Effects of the transient Ca^{2+} concentration on the number (**c**) and velocity (**d**) of sliding actin filaments (F-actin) at various CaM concentrations. Data in **c** and **d** are mean values from three independent experiments.

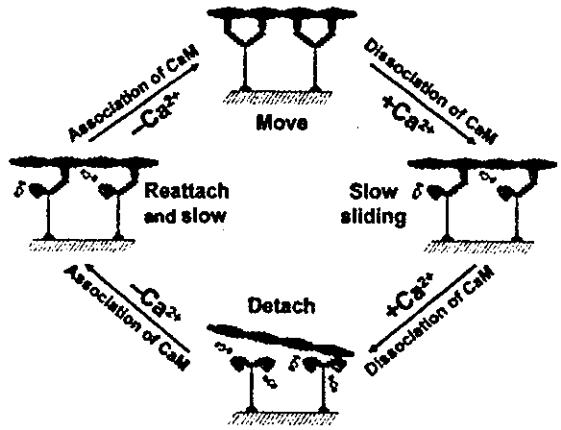

Figure 5 Model for the reversible regulation of myosin V motor functions by Ca^{2+} in the presence of free CaM. In the absence of Ca^{2+} (pCa > 7.0) (move) the actin filaments slide on myosin V with both heads intact. When the Ca^{2+} concentration increased to pCa ≅ 6.0 (slow sliding), one CaM was lost from one of the myosin heads. The single intact head was still able to move the actin filaments by multiple molecules of myosin V but at a velocity ~60% slower than that of native myosin V. When the Ca^{2+} concentration increased to pCa ≤ 5.6 (detach), myosin V lost both CaM from each of the two heads and the actin filaments became detached from myosin V. When the Ca^{2+} concentration returned to the normal low level, free CaM quickly bound to the vacant IQ motif and myosin V quickly recovered to the single-headed active stage (reattach and slow) and the stage of two heads regained their normal function (move). The structure of the motor domain folding back to the neck domain was adapted from a reported structure[27].

DISCUSSION

In the *in vitro* motility assay, actin filaments exhibited a slow sliding velocity at pCa 6.0 and detached at pCa ≤ 5.6. The sliding velocity and number of attached actin filaments could be restored after washing out Ca^{2+} in the presence of free CaM (Fig. 1a–c), but could not be restored after washing out Ca^{2+} in the absence of free CaM. This indicates that the slowing of movement and detachment of actin filaments are due to the dissociation of CaM.

Regulation of myosin V by single CaM

In previous studies, the number of CaM that dissociated from myosin V at certain Ca^{2+} concentrations was estimated by measuring the change in stoichiometry of CaM per heavy chain using a gel scanning method[20,23]. However, the method does not show the distribution of the number of CaM molecules dissociated from an individual myosin molecule. The exact number of CaM that dissociated also could not be estimated owing to the limitations in resolution using this gel scanning method, because the dissociation of a single CaM molecule from a total of 12 bound[24] to myosin V needed to be observed. These limitations were overcome by using single-molecule imaging techniques to evaluate the exact number of single CaM molecules dissociated from individual molecules of a native myosin V at various pCa values. The results showed that the process of CaM dissociation at gradient Ca^{2+} concentrations includes several distinct dissociation events. In a 3-min incubation at pCa 6.0, at least one or two CaM molecules dissociated from each myosin, and at pCa 5.6, two CaM dissociated from every myosin; this is thought to be the maximum number (Fig. 2c). This suggests that one CaM originally dissociated from one heavy chain at a single specific IQ motif, because the myosin V is a homodimer[16]. If this were not the case, the number would be >2. We observed the dissociation of two CaM when myosin V was treated at physiological concentrations of Ca^{2+} (pCa > 5.0)[25], as well as at very high concentrations of Ca^{2+} (pCa 4.5) for 10 s. However, ~15% of the myosin V molecules dissociated three CaM molecules or more when the myosin V was treated at pCa 4.5 for 5 min (data not shown). This agrees with the data from a previous study[26] in which ~2.1 CaM dissociated when CaM was exchanged with *CaM by treatment of myosin V with a solution containing 1 mM EGTA and 1.01 mM $CaCl_2$ (pCa ≅ 4.6) for 5 min. Using the same method, another group of researchers[13] obtained a myosin V mixture containing ~76% of the labeled CaM exchanged at the IQ motif at the end of neck domain connecting to the head of myosin V. Their findings also suggested that a single CaM molecule dissociated from a specific site on the myosin V heavy chain.

The good correlation between the number of sliding actin filaments and the dissociation of single CaM molecules from myosin V suggests that actin-binding affinity is regulated by the dissociation of single CaM molecules. The dissociation of CaM from heavy chains results in a conformational change to the head and neck region of the myosin V molecule[27]. The head-neck domain of myosin V is extended in the absence of Ca^{2+}, whereas at high Ca^{2+} concentrations, the head domain folds back onto the neck domain; this may reduce the actin-binding affinity of the myosin head. The reduction of actin-binding affinity of the myosin head at high Ca^{2+} concentration was also supported by the biochemical results of the actin-activated ATPase activity measured under similar conditions to our motility assay (100 mM KCl; 25 °C). At high Ca^{2+} concentrations, the $K_{m,actin}$ of single-headed myosin V (19.9 μM) is about two-fold higher compared with that in the absence of Ca^{2+} (9.2 μM)[27]. The increase of $K_{m,actin}$ suggests that the actin-binding affinity of the myosin head was reduced at high Ca^{2+} concentrations. This may disrupt the overlap between the two heads of a myosin V, which would reduce the processivity of double-headed myosin V, causing the detachment of sliding actin filament. The high Ca^{2+} concentrations (>1 μM) reduce the actin-binding affinity of the myosin V head through the dissociation of single CaM molecules by binding Ca^{2+} and folding back of the head domain onto the neck. In contrast to glass binding myosin V, in solution the intact myosin V strongly binds to actin filament in the presence of Ca^{2+} (ref. 28); thus the ATPase activity remains high.

We characterized the kinetics of the Ca^{2+}-dependent dissociation of CaM from myosin V at a single-molecule level. In the absence of Ca^{2+}, CaM binds very rapidly and with a high affinity to myosin V, maintaining a stable association of CaM with myosin V for a functional motor at normal low cellular Ca^{2+} concentrations. In the presence of Ca^{2+}, the rapid dissociation of CaM (within seconds) allows myosin V to be regulated by transient Ca^{2+} bursts in cells.

Transient regulation of myosin V

Myosin V is abundantly expressed in nerve cells[15]. It has also been reported that the tips of filopodia (the finger-like projections on the tips of developing nerve cells) generate tiny bursts of Ca^{2+} that travel back to the growth cone to stimulate movement in the correct direction[29]. We simulated the cellular Ca^{2+} bursts by using caged Ca^{2+} coupled with a UV flash system to generate Ca^{2+} transients locally within a time range of a few seconds in an *in vitro* motility assay. We observed a quick response of myosin V motor functions as demonstrated by the detachment of filaments and a reduction in velocity of actin filament sliding during the UV flash. Compared with samples containing 10 or 1 μM CaM, the slower recovery in the case of 0.1 μM CaM and the fact that no recovery occurred in the absence of free CaM support our conclusion that CaM dissociation is involved in the regulation of actin-binding affinity and motility of myosin V. Although the association rate of CaM is very quick in the absence of Ca^{2+} (<1 s at 10 μM CaM) the reattachment of the actin filaments was slower, probably because of the low concentration of actin filaments in the medium.

Model for the regulation of myosin V

We propose a model for the transient regulation of various myosin V functions by Ca^{2+} (Fig. 5). In the absence of Ca^{2+}, the native myosin V has a full complement of bound CaM and moves the actin filaments with both heads. When the Ca^{2+} concentration increased to pCa ≅ 6.0, one of the two heads of myosin V lost one CaM and myosin was in the single-headed active stage. At this stage, the actin-binding affinity was partially reduced and the velocity was slow. Increasing the Ca^{2+} concentration further (pCa 5.6), caused the other head of myosin V to also lose one CaM. Myosin V then moved to the double-headed inactive stage, causing the actin filaments to detach. When Ca^{2+} was chelated, the free CaM quickly rebound to one of the heads that had lost a CaM and myosin V once again was in the single-headed active stage. Finally, the second CaM associated with myosin V and both heads bound a CaM, in what is known as the double-headed active stage of myosin V. In conclusion, the binding of 3–4 Ca^{2+} to one CaM (Fig. 2e) triggers the dissociation of a single CaM molecule from each myosin head, causing a reduction in actin-binding affinity and in the sliding velocity of myosin V. This model of the regulation of myosin V by Ca^{2+} involving various intermediate stages could be useful in understanding how CaM-binding motor proteins bind, move, detach, rebind and move again on actin filament tracks in cells.

METHODS

Chemicals and proteins. Bovine brain CaM was obtained from Sigma. Alexa-647-phalloidin, *o*-nitrophenyl EGTA tetrapotassium salt[30] and Ca^{2+}-sensitive bright Rhod-2 tripotassium salt were obtained from Molecular Probes. Myosin V was purified from chick brains[24]. G-actin was purified from chicken breast muscle[31]. Actin filaments were prepared by polymerization of G-actin in high-salt solution and labeled with Alexa-647-phalloidin. *CaM was prepared as described[26] except that the buffer for labeling was adjusted to pH 7.2 for specific labeling at the N terminus. The obtained *CaM has a Cy3/CaM mole ratio of 1.04:1. *CaM bound to a glass surface in 20 mM HEPES, pH 7.2, was observed as single spots for ~20 s and photobleached in one step, proving that purified *CaM were indeed single molecules.

Buffers and solutions. Buffer A contained 25 mM K-acetate, 100 mM KCl, 4 mM $MgCl_2$, 10 mg ml^{-1} BSA, 1 mM EGTA and 20 mM HEPES, pH 7.2 (pCa > 7.0). EGTA-Ca^{2+} solutions with different free Ca^{2+} concentrations were adjusted by mixing various ratios of buffer A with solution B (buffer A supplemented with 1.05 mM $CaCl_2$). The free Ca^{2+} concentration in the mixture[32] was calculated based on the K_d of EGTA-Ca^{2+}. An oxygen scavenger system[33] was added to all buffers for all imaging experiments.

Single-molecule microscopy. The sealed chambers of all assays were observed under total internal reflection fluorescence microscopy (TIRFM)[33,34]. *CaM and Rhod-2 were excited by a Nd:YAG laser (TIM-6222, Transverse) at 532 nm. Alexa-647-labeled actin filaments were excited by a diode laser (F44-30M, Coherent) at 635 nm. The image was recorded on a DVD recorder (DVR-7000; Pioneer). UV flashes for caged Ca^{2+} activation[35] were created by a 100-W high-pressure mercury lamp, passed through a band-pass filter of 340–370 nm, controlled with a shutter, passed through the objective lens and at last focused into the chamber solution.

Slide quartz glasses, cover glasses and spacers (25-μm thick) were sonicated with 0.1 M KOH and then with 5 M HCl and rinsed with distilled water before use to reduce the background interference for single-molecule imaging and to reduce the effect on the motility activity of myosin V in an *in vitro* motility assay. The chamber was assembled using a slide quartz glass, a cover glass and two spacers. The chamber volume was ~6 μl.

***In vitro* motility assay of myosin V.** The *in vitro* motility assays of myosin V were carried out in the absence and presence of Ca^{2+}. In the presence of Ca^{2+}, the Ca^{2+} concentration was changed in a stepwise or transient manner, depending on the purpose of the experiment (Supplementary Methods online).

Substitution of CaM with Cy3-CaM. A flow glass chamber was rinsed with BSA in 10 μl buffer A, and exchanged with 6 μl of 0.025 nM myosin V in buffer A (~0.19 molecules μm^{-2}) for 2 min to allow the myosin to bind to the glass surface. The slide was then treated with 10 μl of the Ca^{2+} solution for 3 min. The free Ca^{2+} was washed out and the dissociated CaM was substituted with 20 μl of 100 nM *CaM in buffer A for 10 min (10 min was sufficient for most of the dissociated CaM to be substituted (Fig. 3a) and was also enough time for restoration of myosin V motility back to almost 'normal' levels; data not shown). Finally, the free *CaM was washed by incubation with 20 μl buffer A for 3 min. *CaM bound on myosin V was observed under TIRFM after exchange of the buffer with buffer A supplemented with an oxygen scavenger system.

The majority of the dissociated CaM was substituted with *CaM within 5 min (Fig. 3a) and observations could be made for at least 1 h under TIRFM without affecting the number or the intensity of the substituted *CaM fluorescent spots. This indicates that in the presence of 100 nM *CaM and in the absence of Ca^{2+}, the dissociation rate of CaM from myosin V is negligibly slow compared with the association rate (at least ten times slower). The number of substituted *CaM fluorescent spots indicates the number of CaM-dissociated myosin V molecules because almost no spots (0.6% compared with sample in the presence of myosin V) were observed in the absence of myosin V.

For determination of the association and dissociation rate of CaM from myosin V, see **Supplementary Methods** online.

All mentioned assays were done at 25 °C.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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