Myosin VI: cellular functions and motor properties

Rhys Roberts¹, Ida Lister^{1†}, Stephan Schmitz², Matthew Walker³,
Claudia Veigel², John Trinick³, Folma Buss⁴ and John Kendrick-Jones^{1*}

¹MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

²Division of Physical Biochemistry, NIMR, The Ridgeway, Mill Hill, London NW7 1AA, UK

³School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK

⁴Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK

Myosin VI has been localized in membrane ruffles at the leading edge of cells, at the trans-Golgi network compartment of the Golgi complex and in clathrin-coated pits or vesicles, indicating that it functions in a wide variety of intracellular processes. Myosin VI moves along actin filaments towards their minus end, which is the opposite direction to all of the other myosins so far studied (to our knowledge), and is therefore thought to have unique properties and functions. To investigate the cellular roles of myosin VI, we identified various myosin VI binding partners and are currently characterizing their interactions within the cell. As an alternative approach, we have expressed and purified full-length myosin VI and studied its *in vitro* properties. Previous studies assumed that myosin VI was a dimer, but our biochemical, biophysical and electron microscopic studies reveal that myosin VI can exist as a stable monomer. We observed, using an optical tweezers force transducer, that monomeric myosin VI is a non-processive motor which, despite a relatively short lever arm, generates a large working stroke of 18 nm. Whether monomer and/or dimer forms of myosin VI exist in cells and their possible functions will be discussed.

Keywords: myosin VI; actin; molecular motors; membrane trafficking pathways; endocytosis

1. INTRODUCTION

Movement is an essential part of life at every level. From our ability to run and walk, right down to the movement of membranous vesicles in cells, specialized methods of transport have been adapted to help us function as efficiently as possible. For example, within the cell, specific cargo is transported between different localizations using a variety of motor proteins moving along actin and microtubule tracks. It is believed that intracellular cargoes are moved over short distances along actin filaments and over long distances along microtubules to reach their specified intracellular destinations (Langford 1995). The motor proteins that move along microtubules are known as kinesins and dyneins, whereas actin-based transport within the cell is mediated by the myosin superfamily.

2. THE MYOSIN SUPERFAMILY

Myosins are actin-activated Mg^{2+} ATPases that use energy derived from ATP hydrolysis to generate force and move along actin filaments. All myosins contain a well-conserved domain organization consisting of an N-terminal motor domain, a neck region (lever arm) containing from one to six IQ motifs that bind light chains or calmodulin, and a highly variable tail domain. The motor domain is responsible for

binding and moving along actin filaments. In contrast, the tail domain binds cargo and has been predicted to target particular myosins to specific cellular compartments and thus determine their function. By comparing the amino acid sequences of their motor domains, the myosins can be divided into at least 18 distinct classes (Hodge & Cope 2000; Berg *et al.* 2001). Interestingly, an identical classification is observed if the sequences of their accompanying tail domains are compared, implying that the motor and tail domains have evolved together so that a motor domain has specific kinetic properties when linked to a tail domain in order to perform distinct functions in the cell. In skeletal muscle, the class II myosin is specialized for one main function (i.e. muscle contraction), whereas it has become apparent that the other classes of myosins in non-muscle cells may have multiple functions.

The class I and IX myosins are monomers, whereas the class II and V myosins are dimers. So what determines whether a myosin is a monomer or a dimer? At the primary level this is decided by the presence of a dimerization interface such as a region capable of forming a stable α -helical coiled coil. In addition, the functions that they perform within the cell may specify whether a class of myosins exists as a monomer or dimer and this may be determined by the cargo molecule that is bound. An intriguing question is whether a 'multifunctional' myosin might be a monomer for certain functions and then assemble to form a dimer to perform alternative roles in the cell.

Now that various genome sequencing projects have been completed, we are able to catalogue the myosins present in

[†] Present address: Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA.

* Author for correspondence (jkj@mrc-lmb.cam.ac.uk).

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Figure 1. (a) The domain organization of myosin VI. The N-terminus contains the motor domain with the ATPase and actin binding sites involved in movement and force generation, followed by the lever arm composed of the unique 53 amino acid insert (orange) and a single IQ motif (yellow), both of which bind calmodulin. The helical region of the tail (turquoise) is followed by the so called globular C-terminal end of the tail (blue) involved in binding cargo molecules, such as membrane receptor binding proteins. (b) Myosin VI moves towards the minus end of actin filaments. It is believed that actin filaments in cells have their plus ends (+) inserted into or near membranes (Cramer 1999), as shown in this diagram. Given this actin filament polarity, myosin VI would move along actin filaments away from the plasma membrane into the cell and away from the surface of intracellular organelles such as the Golgi complex. The red lines outline a clathrin-coated vesicle being formed at the plasma membrane and then being moved by a myosin VI molecule into the cell (Buss *et al.* 2001). Non-clathrin-coated vesicles are also moved around the cell by myosin VI (Aschenbrenner *et al.* 2003).

the following organisms (Vale 2003); humans possess 40 different myosin genes encoding 12 different classes (Berg *et al.* 2001), yeast (*Saccharomyces cerevisiae*) contains five genes encoding myosins from three classes (Brown 1997) and *Drosophila melanogaster* appears to contain 13 genes encoding myosins from eight classes (Yamashita *et al.* 2000; Tzolovsky *et al.* 2002). Interestingly, plants contain multiple members of just two classes of myosins: the unique class VIII and the class XI myosins (which are very closely related to the 'universal' class V myosins; Berg *et al.* 2001; Hodge & Cope 2000). As more genome sequences are completed, further novel myosins may be identified. However, despite the large number of myosins identified in many organisms, the precise roles that most of them play within cells are not yet known.

Most of our information on the mechanism of movement along actin filaments has been gained from extensive studies on the muscle myosin II motor domain (Geeves & Holmes 1999). The crystallographic structures of the

motor domain of myosin II in the presence and absence of different nucleotide analogues have recently been solved and have formed the basis for a model for myosin movement known as the 'lever arm' model (Rayment *et al.* 1993; Smith & Rayment 1996; Houdusse *et al.* 1999). Basically, this model predicts that small conformational changes in the motor domain, as a result of ATP hydrolysis and interaction with actin, are amplified by the converter region at the end of the motor domain causing the neck region (lever arm) to swing and this results in movement (Holmes 1997). Although this lever arm mechanism is thought to operate in the class II and V myosins, whether it operates in all classes of myosins has yet to be established.

The C-terminal tail domains are very poorly conserved between different classes of myosins and, apart from the class II myosins which assemble to form filaments, much less is known about these domains. Tail domains are thought to determine the function of a myosin by providing an anchor for its motor activity and by interacting with particular binding partners, with the result of targeting it to specific intracellular compartments and multi-protein complexes. Myosin V, for example, has been shown to be a processive dimeric motor (Mehta *et al.* 1999) that can walk along an actin filament, taking many consecutive steps without dissociation. In yeast myosin V is involved in transporting organelles from the mother to the daughter (bud; Bretscher 2003) and in vertebrates it transports pigment-containing melanosomes to keratinocytes and organelles such as the endoplasmic reticulum along neurons (Wu *et al.* 2002b). Mutations in myosin V lead to a loss of pigmentation and neurological defects in mice (Dilute) and in humans (Griscelli's disease) (Reck-Petersen *et al.* 2000; Hume *et al.* 2002). Myosin I, however, is a monomeric non-processive motor that has been shown to be required for the maintenance of membrane tension (Mermall *et al.* 1998). These examples also illustrate how the properties of the motor domain (monomer or dimer, processive or non-processive) need to be modulated to be in tune with the function specified by the tail. Thus the different classes of myosins are thought to have a wide range of roles in the different types of cells present in an organism. In this paper we focus on our work to elucidate the intracellular roles of one of these classes of myosin, namely myosin VI.

3. MYOSIN VI

Myosin VI was first discovered in *Drosophila* as a 140 kDa ATP-sensitive actin binding protein, and upon further analysis it was revealed as the first member of a new class of myosins, class VI (Kellerman & Miller 1992). Its amino acid sequence indicates that it follows the basic myosin domain organization, with an N-terminal motor domain followed by a neck region containing a single IQ motif and a C-terminal tail domain. Within the motor domain there is a unique 22 amino acid insert near the ATPase site which may be involved in controlling the release of nucleotide from the nucleotide binding pocket. Between the motor domain and neck region there is an additional unique 53 amino acid insert which has been predicted to be the 'reverse gear' that allows myosin VI to move towards the minus end of actin filaments (Wells *et al.* 1999). Unexpectedly this insert has recently been shown to bind calmodulin (Bahloul *et al.* 2004) despite not containing a recognizable IQ-calmodulin

Figure 2. Localization of myosin VI in fibroblasts. (a) Endogenous myosin VI was localized by immunofluorescence microscopy using a polyclonal antibody raised against the globular tail region of myosin VI. (b) The expression of GFP-tagged myosin VI gives a very similar localization of myosin VI in these cells. They show that myosin VI is localized in the Golgi complex in the perinuclear region of the cell, in membrane ruffles at the leading edge of the cell and is present in vesicles distributed throughout the cell. The identity of these cellular locations was confirmed by co-localization using known markers to these cellular compartments (Buss *et al.* 1998). Scale bar, 10 μ m.

binding motif. Figure 1a shows a diagram of the domain structure of myosin VI. When the tail sequences of myosin VI were first analysed they were predicted to contain a region of coiled coil and therefore to exist as a dimer (Kellerman & Miller 1992; Hasson & Mooseker 1994). However, more recent sequence and biophysical analyses question whether myosin VI exists as a stable dimer (Lister *et al.* 2004).

Myosin VI is ubiquitously expressed in higher eukaryotes (Sellers 2000). It is essential for hearing and for the development and maintenance of the stereocilia in the hair cells in the inner ear in mice (Self *et al.* 1999) and for spermatogenesis, oogenesis and cell motility in *D. melanogaster* and

Caenorhabditis elegans (Geisbrecht & Montell 2002). Myosin VI exists in several different isoforms with inserts in the tail region, which are splice variants derived from a single gene (see figure 8a). The actual number of isoforms expressed differs between organisms; in *Drosophila* there are at least three myosin VI isoforms (Kellerman & Miller 1992), whereas in mammalian cells four isoforms are expressed (Buss *et al.* 2001). These isoforms are expressed in a tissue-specific manner; for example, in mammalian cells the isoform with the large tail insert (*ca.* 31 residues) is expressed in tissues with polarized epithelial cells containing apical microvilli, such as kidney and small intestine, whereas the isoforms with the small insert (nine residues) or with no insert are expressed in tissues containing non-polarized cells, such as testis and lung. Experiments using the human brush border Caco-2 cell line indicate that a single cell type can change its myosin VI expression pattern (Buss *et al.* 2001). For example, if Caco-2 cells are grown on a plastic surface, which prevents the cell from polarizing into apical and basolateral domains, then the myosin VI isoform with the small or no insert is expressed; however, when grown on membrane filters that allow the cells to become maximally polarized then these same cells now switch to expressing myosin VI with the large insert. Although it is the C-terminal globular region of the tail that contains the binding sites for myosin VI interacting proteins, the presence of the large insert also appears to be necessary for myosin VI involvement in clathrin-mediated endocytosis in polarized epithelial cells (Buss *et al.* 2001).

In 1999, one of the most fascinating properties so far discovered about myosin VI was reported by Wells *et al.* (1999). Using a construct containing the motor domain and neck region of myosin VI they showed that this myosin moved towards the minus end of actin filaments in an *in vitro* motility assay—in the opposite direction to all of the other so far characterized myosins. Interestingly, because it is believed that the plus ends of actin filaments are positioned towards membranes (Cramer 1999), then myosin VI's motion towards the minus end of actin filaments would cause movement towards the centre of the cell, away from the plasma membrane and away from the surface of intracellular organelles such as the Golgi complex (figure 1b). Thus myosin VI as a minus-end-directed motor would be expected to have unique cellular functions and mechanical properties. The unique 53 amino acid insert in myosin VI between the converter region in the motor domain and the IQ motif in the neck (termed the 'reverse gear') is believed to be responsible for movement in the reverse direction along actin (Wells *et al.* 1999). Interestingly, recent molecular engineering studies (Tsiavaliaris *et al.* 2004) have shown that a plus-end-directed myosin can be converted into a minus-end-directed motor by inserting a specific structural domain into the neck region. Whether nature has adopted a similar procedure to reverse the direction of movement of a myosin in the cell, by for example, adding or binding an extra component to the neck region, will be a fascinating question to explore.

In mammalian cells, myosin VI has been shown to be involved in endocytosis, in membrane ruffling at the leading edge of cells and in the maintenance of Golgi complex morphology and in secretion. Although cellular localization studies (see figure 2) have confirmed that myosin VI is present in clathrin-coated and uncoated vesicles dis-

Table 1. The myosin VI binding partners so far identified can be grouped together according to their cellular localizations and potential functions.

location/cellular function	binding protein	references
plasma membrane/receptor mediated endocytosis	Dab2	Morris <i>et al.</i> (2002)
	SAP97 (part of the synapse AMPA receptor)	Wu <i>et al.</i> (2002a)
	GIPC	Bunn <i>et al.</i> (1999)
Golgi complex/morphology and secretion	FIP2 (optineurin)	
cell motility	Golgin-like protein	
	BAL/Rhysin 1	
	<i>Drosophila</i> -CLIP 190	Lanz & Miller (1998)
	<i>Drosophila</i> E-cadherin/ β catenin	Geisbrecht & Montell (2002)

tributed throughout the cell (Buss *et al.* 2001; Aschenbrenner *et al.* 2003), in membrane ruffles (Buss *et al.* 1998) and in the Golgi complex network (Warner *et al.* 2003), little is known about its precise role(s) in these locations. To gain insights into how myosin VI functions in a variety of cells, we have used a number of approaches.

4. IDENTIFICATION OF MYOSIN VI BINDING PARTNERS

(a) *Yeast two-hybrid system*

The yeast two-hybrid system has been used successfully to identify both known and previously unknown binding partners (Stephens & Banting 2000). Its strength lies in its ability to test many potential interacting proteins in a single experiment. We used the myosin VI tail domain, which is the region involved in cargo binding, as bait to screen a cDNA library (from human umbilical vein epithelial cells) for interacting protein using the yeast two-hybrid system. However, we immediately encountered a major problem. The tail domain, especially the globular cargo-binding region used as the bait, self activated (i.e. it activated transcription of the reporter gene autonomously). Because the yeast two-hybrid system relies on the detection of reporter gene transcription only when interacting proteins bind together, bait proteins that auto-activate cannot normally be used. However, we found that if we used the Pro-Quest two-hybrid system (Invitrogen), we could inhibit, in a dose-dependent manner, the self activation by the tail domain (see Pro-Quest instruction manual catalogue series 10835; see http://invitrogen.co.jp/products/pdf/custom_proquestlibrary.pdf), thus allowing it to be used as a bait for screening for interacting proteins.

Another major problem encountered using the yeast two-hybrid system is the possibility of generating 'false positives'. These are proteins that appear to interact with the bait in the yeast system but do not truly interact in their native environment, and are thus biologically irrelevant. We found that by screening the proteins identified in the yeast screen using a mammalian two-hybrid system (Morris *et al.* 2002) we were able to eliminate 'false positives' and identify 'authentic' myosin VI binding proteins.

A list of the proteins so far identified by us and other groups as myosin VI binding proteins is shown in table 1. They can be divided roughly into three groups: proteins at the plasma membrane involved in endocytosis, those at the Golgi complex and those associated with cytosolic vesicles. These cellular compartments are where myosin VI has

previously been shown to be localized (Buss *et al.* 1998; figure 2).

(b) *Immunoprecipitation and mass spectrometry*

As another approach to identify proteins that associate with myosin VI *in vivo* we used immunoprecipitation with myosin VI tail antibodies to isolate myosin VI-containing complexes from cells. The protein components in these complexes were separated by SDS-PAGE, and after in gel tryptic digestion the resulting peptides were subjected to matrix-assisted laser desorption ionisation (MALDI) peptide mass mapping (Jensen *et al.* 1999), and the proteins identified by searching the peptide mass databases. In several cases, however, no matches in the databases were obtained suggesting that they were novel proteins. Selected peptides from these unidentified proteins were thus sequenced by quadrupole time-of-flight (Q-tof) mass spectrometry and the sequence information used to search online databases to identify them: one of these novel proteins was named Rhysin 1. When the human genome sequencing project had been completed we were able to locate the gene coding for this protein on chromosome 3q21. The GENSCAN gene prediction program (Burge & Karlin 1997) was used to predict its sequence. At the same time that Rhysin 1 was cloned, an identical protein named BAL (Aguiar *et al.* 2000) was identified. Work is in progress to characterize this protein and verify its association with myosin VI.

This work provides a good example of the potential power of these approaches for detecting and identifying hitherto unknown biological interactions. However, this is only just the first stage and the interactions of myosin VI with its newly identified binding partners need to be confirmed using additional techniques, such as co-localization, co-immunoprecipitation and 'pull down' assays. To illustrate these procedures we will describe our work characterizing the interaction of myosin VI with one of its binding partners, Dab2 (DOC-2), identified by a yeast-2-hybrid screen (Morris *et al.* 2002).

(c) *Co-localization, co-immunoprecipitation and 'pull down' assays*

Dab2 was initially identified as a negative regulator of cell growth and was found to downregulate several different cancers. It has now been shown to be involved in the regulation of several signalling pathways such as the Ras/mitogen-activated protein kinase (Ras/MAPK) (Xu *et al.* 1998) and the Wnt signalling pathways (Hocavar *et al.*

Figure 3. Dab2 is a potential linker molecule for cell signalling and endocytosis. In its N-terminal domain it contains a phosphotyrosine domain (PTB) which binds to the internalization signal (NPxY) in the cytoplasmic tails of plasma membrane receptors of the LDLR family, for example megalin. In its central region, Dab2 contains a DPF (aspartic acid, proline, phenylalanine) binding motif which binds to the α -ear of the adaptor protein AP2 involved in clathrin-mediated endocytosis and five NPF (asparagine, proline, phenylalanine) binding motifs for proteins such as Eps15 associated with the endocytic machinery. At its C-terminal it contains the SYF (serine, tyrosine, phenylalanine) motif, which binds to the globular tail (cargo-binding) region of myosin VI and an Src homology domain 3 (SH3) domain that binds to the growth factor receptor-bound protein 2 (Grb2).

2001). Dab2 is a 96 kDa protein with several known signalling domains and motifs (figure 3). At its N-terminus it contains a phosphotyrosine domain that binds to the NPxY internalization signal in the cytoplasmic tails of LDLRs and is believed to be involved in endocytosis of these LDLRs (Morris & Cooper 2001). Dab2 also contains sites for binding to the adaptor protein AP2, to clathrin and to other endocytic accessory proteins such as Eps15 (see figure 3), suggesting that it plays a role in clathrin-mediated endocytosis by acting as an 'adaptor protein' linking plasma membrane receptors to the endocytic machinery, and by binding to myosin VI links to the actin cytoskeleton (Morris & Cooper 2001; figure 4b).

'Pull down' assays using GST-myosin VI tails showed that myosin VI bound to endogenous Dab2 is present in fibroblast cell lysates (Morris *et al.* 2002). Co-immunoprecipitation of Dab2–myosin VI complexes from Caco-2 cells using Dab2 antibodies further confirmed that Dab2 binds to myosin VI. Double labelling in polarized Caco-2 cells for myosin VI and Dab2 showed significant co-localization, especially in clathrin-coated vesicles and pits at the apical surface of these cells (figure 4a). Interestingly, when GFP-labelled myosin VI is transfected into fibroblasts it only co-localizes with Dab2 in clathrin-coated vesicles if the specific myosin VI isoform with the large insert was used (Buss *et al.* 2001). These studies also showed that overexpressing the non-functional tail domain of myosin VI (missing the motor domain) in fibroblasts significantly reduced (by 60%) clathrin-mediated endocytosis in these cells. This was the first finding, to our knowledge, of a motor protein being associated with clathrin-mediated endocytosis.

5. ROLE OF MYOSIN VI IN ENDOCYTOSIS?

So what role(s) does the myosin VI–Dab2 complex perform in endocytosis at the plasma membrane? Clathrin-mediated endocytosis is the best-characterized pathway for the uptake of cell surface receptors and involves the

major players clathrin and the adaptor protein AP2, together with a whole host of accessory proteins such as membrane binding proteins, structural coat components and actin binding proteins (Brodsky *et al.* 2001; figure 5). The steps in the pathway involve: (i) the initial selection of the cargo and receptors, and their clustering in the membrane; (ii) the formation of the CCP and spatial organization leading to clathrin coat assembly; (iii) invagination of the plasma membrane, membrane scission and formation of a clathrin-coated vesicle; and (iv) transport of the vesicle into the cell. Myosin VI, by binding to Dab2, could link plasma membrane receptors to the actin cytoskeleton and, by moving towards the minus end of the actin filament, could be involved in several steps in clathrin-mediated endocytosis such as: clustering of plasma membrane receptors; providing the force for plasma membrane invagination and vesicle formation and the short-range movement of vesicles along actin filaments away from the plasma membrane. Dissecting the mechanisms involved will be a great challenge and may provide a generalized model for receptor-mediated endocytosis, including the role played by myosin VI.

Myosin VI has been shown by yeast-2-hybrid screens to bind to other adaptor molecules such as GIPC (Bunn *et al.* 1999; Gao *et al.* 2000) and SAP97 (Wu *et al.* 2002a) that can link myosin VI to receptors of the LDLR family (table 1). GIPC binds to the cytoplasmic tails of LDLRs such as megalin at the plasma membrane in the gut and has been shown to co-localize with myosin VI in clathrin-coated vesicles (Lou *et al.* 2002) and in uncoated vesicles in transit to the endocytic compartment (Aschenbrenner *et al.* 2003). SAP97 binds to the Glu1 subunit of the AMPA-glutamate receptors in brain synapses and is thought to have a role in the delivery of newly synthesized AMPA receptors via the Golgi complex to the plasma membrane (Sans *et al.* 2001). Myosin VI and SAP97 can be co-immunoprecipitated as a complex from a rat brain extract (Wu *et al.* 2002a), suggesting a potential role for myosin VI in AMPA receptor trafficking.

6. MYOSIN VI 'KNOCK-OUT' MOUSE (SNELL'S WALTZER MOUSE)

The *sv* mouse has been used primarily as a model for human deafness (Avraham *et al.* 1997) and displays the typical physical signs of inner ear defects such as head tossing, circling, hyperactivity and, of course deafness. In the *sv* mouse, a spontaneous mutation was found to cause a large intragenic deletion in the myosin VI transcript resulting in a frameshift and subsequent stop codon at the beginning of the neck region. No myosin VI could be detected in these mice so they are referred to as a myosin VI null mutant (Avraham *et al.* 1995).

In normal mice, in the cochlear hair cells in the inner ear, myosin VI is found at the base of the actin-rich stereocilia, at the cuticular plate and in the pericuticular necklace in the apical domain (Hasson *et al.* 1997). Sound is detected by small movements of these specialized stereocilia. In the developing *sv* mouse, the cochlear hair cells appear normal at birth, but 3 days later the stereocilia become disorganized and start to fuse to form giant stereocilia protruding from the apices of these cells (Self *et al.* 1999). These authors propose that myosin VI may be important in

Figure 4. (a) Myosin VI interaction with Dab2 confirmed by co-localization in fibroblasts. Expressed GFP-tagged myosin VI partially co-localizes with endogenous Dab2 in clathrin-coated vesicles in these fluorescent micrographs. This interaction was further confirmed by 'pull-down' assays using GST-tagged myosin VI tails which pelleted Dab2 from cell cytosol, co-immunoprecipitation of myosin VI-Dab2 complexes again from cell cytosol using antibodies against Dab2, and mammalian-2-hybrid screens (Morris *et al.* 2002). (b) A diagram illustrating how the Dab2-myosin VI complex might form a dynamic link between cell surface receptors such as megalin on the plasma membrane, the endocytic machinery via the adaptor protein AP2 and the actin cytoskeleton. The phosphotyrosine domain (PTB) in Dab2 binds to the internalisation signal (NPxY) in the cytoplasmic tail of the plasma membrane megalin receptor.

anchoring the plasma membrane by pulling it towards the centre of the hair cell (towards the minus end of the actin filaments), thereby preventing the stereocilia from fusing and becoming the giant structures observed in the inner ear of the *sv* mouse. Preliminary observations indicate that the microvilli in the brush border cells in the intestine are also significantly reduced in size in *sv* mice (Self *et al.* 1999). Therefore there must be a large degree of redundancy in the mouse for it to survive without myosin VI. However, a careful widespread characterization of the *sv* mouse has not yet been carried out to identify other phenotypes that may occur because of the absence of myosin VI.

To try to rectify this omission we have prepared primary fibroblasts and immortalized fibroblastic cell lines from the *sv* mouse and started with characterize and compare them with cells from wild-type mice (Warner *et al.* 2003). The most apparent morphological difference is the size of the Golgi complex, which is significantly reduced by *ca.* 40% in the *sv* fibroblast compared with the wild-type cells. Previous studies (Buss *et al.* 1998) had shown that myosin VI was localized at the Golgi complex in mammalian cells (figure 2). In these higher eukaryotic cells the Golgi complex exists as a stack of flattened membrane cisternae composed of the *cis*, medial and *trans* Golgi compartments, localized in a perinuclear position (Ladinsky *et al.* 1999). The Golgi complex is known to play a central role in the cell because every protein synthesized in the endoplasmic reticulum

passes through it, undergoing post-translational modification and subsequent sorting for transport to its final destination (figure 5). Interestingly, in the *sv* fibroblasts, where myosin VI is missing, the level of secretion from the Golgi complex to the plasma membrane was also reduced by 40% (Warner *et al.* 2003). When wild-type myosin VI was overexpressed in these cells, both Golgi morphology and secretion were rescued to wild-type levels. If constructs containing only the tail domains (without the motor domain they are non-functional) were overexpressed, neither of these phenotypes was rescued.

So what possible functions could myosin VI have at the Golgi complex? The reduction in the size of the Golgi complex in its absence suggests that myosin VI must play a dynamic role linking the Golgi membrane to the surrounding actin cytoskeleton, so as to maintain the extended characteristic morphology of the Golgi stacks. Myosin VI is also involved in secretion; it may be required for vesicle formation and/or for the sorting of the different cargo proteins into vesicles and their short-range transport towards different cellular destinations.

7. CELL MOVEMENT

Myosin VI has been shown to be present in the dynamic membrane ruffles that occur at the leading edge of moving cells (Buss *et al.* 1998; figure 2). When mammalian cells are stimulated by growth factors such as epidermal growth fac-

Figure 5. A diagram illustrating the major steps in clathrin-mediated endocytosis and exocytosis in a mammalian cell. In endocytosis at the plasma membrane, initially the receptors (cargo) are selected and clustered together, possibly involving myosin VI, and then clathrin, the adaptor protein AP2 and a whole host of accessory proteins assemble together to form a CCP. The plasma membrane invaginates (myosin VI acting with the actin cytoskeleton may pull in the membrane?) then membrane fission occurs (again with a large number of coat components, accessory proteins, myosin VI and the actin cytoskeleton involved), and a clathrin-coated vesicle is formed (CCV) which moves into the cell, uncoats and is transported to early and late endosomes, again possibly involving myosin VI? Similarly at the trans-Golgi network in the Golgi complex, cargo molecules are sorted and assembled into secretory vesicles (involving myosin VI?) which are transported (by myosin VI?) to the plasma membrane. So which of these steps in endocytosis and exocytosis involves myosin VI?

tor, myosin VI is actively recruited into membrane ruffles and there is a three to fourfold increase in phosphorylation in the motor domain, possibly mediated by a PAK, a member of the G-protein rac and cdc42 activated kinase family (Buss *et al.* 1998). PAKs are known to be involved in regulating cell motility in fibroblasts (Sells *et al.* 1999). The site predicted to be phosphorylated in the motor domain was T⁴⁰⁶ in the so-called 'cardiomyopathy loop' in the actin binding interface. This site obeys the TEDS (threonine (T), glutamic acid (E), aspartic acid (D), serine (S)) rule for myosin motor domain phosphorylation sites proposed by Bement & Mooseker (1995). Using a phospho-specific antibody generated against phospho-threonine 406, we were able to confirm that myosin VI phosphorylated at this site is present in the membrane ruffles. Using GFP-tagged myosin VI deletion mutants we demonstrated that both motor and tail domains are required for recruitment into ruffles (Buss *et al.* 1998). At present, however, the precise role(s) of myosin VI in membrane ruffling and cell motility is not known. At the leading edge of moving cells, actin polymerization is believed to drive the formation of long finger-like projections, such as lamellipodia and filopodia, which extend outwards and adhere to the substrate and are believed to provide the protrusive force to power the cell forwards (Mitchison & Cramer 1996; Hall 1998). In *Drosophila*, myosin VI is essential for the migration of border cells in the ovary

(Geisbrecht & Montell 2002). In these cells myosin VI forms a complex with β catenin and E-cadherin, which form adhesion complexes in the membrane that bind to the substrate and are required for forward movement. Myosin VI, by binding to these stationary adhesion complexes in the membrane and moving towards the minus end of the newly formed actin filaments, could also develop a protrusive force for pushing these actin filaments forwards (Geisbrecht & Montell 2002). Our current work is aimed at determining whether, in mammalian cells, myosin VI binds to the cell adhesion proteins and is involved in coordinating cell adhesion, membrane protrusion and forward cell movement.

8. WHAT TYPE OF MOTOR IS MYOSIN VI?

What type of motor protein would myosin VI need to be to carry out the specific functions that it is required to perform in the different regions in the cell? For all of its potential activities a 'high duty ratio' motor (one that spends most of its ATPase cycle attached to actin) is essential. When myosin VI was first cloned and sequenced it was assumed to be a dimer with two heads (motor domains) because it contained a region in its tail that was predicted to form a coiled coil (Kellerman & Miller 1992; Hasson & Mooseker 1994). It is probably for this reason that most of the *in vitro* motility studies have been carried out on artificial myosin VI dimers where dimerization has been ensured by the presence of a C-terminal leucine zipper or smooth muscle myosin II rod domain (Rock *et al.* 2001; Nishikawa *et al.* 2002). Kinetic and motility measurements indicate that these constructs have a high duty ratio (they are strongly bound to actin for *ca.* 90% of their ATPase cycle; De La Cruz *et al.* 2001) and they move processively along actin filaments with a large step size of *ca.* 30 nm (Rock *et al.* 2001; Nishikawa *et al.* 2002). Recent kinetic data (Morris *et al.* 2003) indicate that calcium binding to the calmodulin in the neck region leads to a loss of coordination between the two 'heads' in the dimer constructs, and as a result processivity is lost and sliding velocity is reduced threefold. All of these studies, however, were carried out on myosin VI constructs that were missing the important 'cargo binding' tail. We therefore expressed and purified the unmodified full-length myosin VI to determine its properties.

9. IN VITRO PROPERTIES OF FULL-LENGTH MYOSIN VI

Unexpectedly, the full-length myosin VI expressed using the insect-baculovirus system behaved as a monomer on gel filtration and sucrose density gradient centrifugation (Lister *et al.* 2004). Using the Stokes radius (6.0 nm) and sedimentation coefficient (6.3 S) measured by these procedures allowed us to calculate a MW value of 153 kDa (close to the theoretical MW value for a monomer of 164 kDa), confirming that our myosin VI is a monomer. Interestingly, native myosin VI prepared from cytosol extracted from normal rat kidney (NRK) fibroblast cells behaves identically to the expressed protein and is a monomer. Cross-linking studies using the zero length cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) confirmed that both our native and expressed myosin VI are monomers (Lister *et al.* 2004).

Perhaps the most convincing evidence that our myosin VI is a monomer is its direct visualization in the electron microscope after negative staining (figure 6). Single particle image processing of the stained molecules shows a large globular region 8–9 nm long that is likely to be the motor domain, followed by a smaller region that may be the neck region with its bound calmodulins, and then another small region which maybe the tail of the myosin (Lister *et al.* 2004). Interestingly, on addition of ATP the molecules undergo a gross conformational change; for example, in the presence of ATP the molecules are straight with an overall length of *ca.* 17 nm, whereas the apo molecules (no nucleotide present) appear to be 20% shorter and have a strongly bent neck–tail region which appears to be folded back under the motor domain (Lister *et al.* 2004; figure 6c).

In vitro motility assays were done on our full-length myosin VI, which had been anchored to the surface of the flow cell using antibodies directed against the tail region (Lister *et al.* 2004). The velocities measured (*ca.* 70 nm s⁻¹) were comparable to those previously reported for single motor domain constructs (Morris *et al.* 2003). Preliminary data obtained at ever-decreasing myosin VI concentrations were consistent with the behaviour of a non-processive motor (Howard *et al.* 1989). Single molecule myosin VI interactions with F-actin measured using an optical tweezers-based force transducer produced a working stroke of *ca.* 18 nm (Lister *et al.* 2004). Only single isolated interactions of the myosin VI with F-actin (attachment events) were observed. When the attachment events were averaged (Veigel *et al.* 2002), it showed that myosin VI, like a number of other myosins (Veigel *et al.* 1999, 2002), produces its working stroke in two steps; a first step of *ca.* 17 nm associated with phosphate release and a second small step of *ca.* 1 nm associated with ADP release, leading to the nucleotide-free rigour state.

10. HOW DOES MYOSIN VI PRODUCE SUCH A LARGE WORKING STROKE?

How is myosin VI, with such a small lever arm, able to produce such a large working stroke? In previous single molecule mechanical experiments on the artificial dimeric myosin VI constructs (Rock *et al.* 2001; Nishikawa *et al.* 2002) processive movements with a large step size of *ca.* 30 nm were observed. This large step size is difficult to explain if only the classical lever arm mechanism is involved because myosin VI, with a lever arm composed of one IQ motif and the 53 residue insert, would only be able to generate a lever arm movement of *ca.* 8–10 nm at most. Several models have been presented to try to explain how such a large step size could be achieved. One model (Rock *et al.* 2001) recently refined by Altman *et al.* (2004) proposes that the large step size in their myosin VI dimer is a result of a working stroke of *ca.* 10 nm in the lead head attached to actin followed by a *ca.* 20 nm diffusive search by the free trailing head for the next available binding site on the actin filament. They speculate that the first part of the tail region is flexible and extended to allow the large diffusive search by the trailing free head. However, Nishikawa *et al.* (2002) propose that the binding of the first head of their myosin VI dimer to actin causes a conformational change in the actin filament that allows the second head to slide and bind *ca.* 30 nm along the filament.

In our case, how can a working stroke of *ca.* 18 nm be generated with a single myosin VI head? We propose that the size of the working stroke of our myosin VI depends not only on the length of the lever arm but also on the orientation and rotation of the lever arm relative to the actin filament (Lister *et al.* 2004; figure 7). Cryo-EM studies on myosin VI bound to actin (Wells *et al.* 1999) showed that in ADP, the orientation of the lever arm pointed *ca.* 65° towards the minus end of the actin filament, whereas in rigor (on ADP release) it pointed even further at *ca.* 85° towards the same end. In myosin VI the two calmodulin binding domains might be able to contribute *ca.* 10 nm to the lever arm. In order for myosin VI with a 10 nm lever arm to produce the first step of 17 nm observed in the optical trap (associated with Pi release), finishing in the ADP orientation (65°), it would need a starting pre-powerstroke lever arm orientation of –53°. The movement of the lever arm from its 65° ADP to its 85° rigor orientation would account for the second step of 1 nm. In total, the swing of the lever arm would then be 138° (Lister *et al.* 2004; figure 7). The sharply bent appearance of the lever arm–tail region in the EM images of myosin VI in rigour (figure 6) would support this model. However, at this stage, all of the models proposed to explain the step size of myosin VI are somewhat speculative, and as a first priority we need to determine the structure of its tail domains.

11. IS MYOSIN VI A MONOMER OR A DIMER IN CELLS?

Our cellular localization and functional studies suggest that myosin VI has multiple roles in cellular compartments such as the plasma membrane and Golgi complex (Buss *et al.* 1998, 2001; Warner *et al.* 2003). Our *in vitro* motility and biophysical results (Lister *et al.* 2004) show that the full-length myosin VI molecule is a monomer and acts as a non-processive motor, whereas previous studies (Rock *et al.* 2001; Nishikawa *et al.* 2002) have shown that artificially dimerized myosin VI motor domain constructs are capable of processive movement. This raises the intriguing possibility that myosin VI may be able to function as a monomer and may be induced to form a dimer *in vivo*. While certain functions such as those involving maintenance of the Golgi complex structure (Warner *et al.* 2003) might require a non-processive monomer, other functions such as transport of endocytic vesicles (Buss *et al.* 2001) might benefit more from a processive dimer (table 1). In these cases it is conceivable that the binding of myosin VI to one of its binding partners, or a post-translational modification of myosin VI such as phosphorylation, might cause dimerization at the site of action such as on a lipid membrane. The idea that dimerization can promote processivity either via a leucine zipper, by binding partners, or through intracellular localization by affecting the conformational and oligomeric state of a motor protein has been experimentally explored using the Kif1/Unc104 kinesin family (Tomishige *et al.* 2002). This kinesin family comprises mainly monomeric motor proteins, but when concentrated on membrane surfaces they can dimerize through a region of coiled coil next to the motor domain and the resulting dimer can move processively along microtubules. A similar scenario might be envisaged for myosin VI, for example, during endocytosis at the plasma membrane when myosin VI is recruited to CCPs, or to budding

Figure 6. Myosin VI can exist as a monomer. (a) An electron micrograph showing a field of negatively stained myosin VI molecules in the presence of 100 μ M ATP. Only monomers are observed. (b) and (c) Image averages after alignment and classification by single-particle processing of the negatively stained myosin VI molecules (b) in the presence of 100 μ M ATP, and (c) in the absence of nucleotide. The motor domain is presumed to be the large region at the top of the molecules and this is followed by the neck region with its bound calmodulins and then the tail. Note the bent appearance of the tail in the molecule in the absence of nucleotide. Scale bar, 10 nm.

or internalized vesicles by one of its binding partners, such as Dab2 (Morris *et al.* 2002) at sufficient concentrations for dimerization to take place via a part of the helical tail domain. The ability to control whether myosin VI exists in a monomer or dimer state could be an important way to regulate the multiple functions of myosin VI in the cell. However we should introduce a word of caution because as yet no native myosin VI dimer molecules have been detected.

12. MYOSIN VI REGULATION

At present we know very little about how the microtubule-based motors, the kinesins and dyneins and the actin-based motors, the myosins, are regulated in the cell. Within the cell these motor proteins do not work in isolation but must communicate with each other to ensure that the dynamic organization of the cell is maintained. Thus the activities of the motors that move towards the plus end and those that move towards the minus end of their microtubule or actin filament tracks must be regulated and coordinated to maintain the correct balance and distribution of organelles and components within the cell. Because myosin VI is the only myosin so far identified as moving towards the minus end of actin filaments and given its wide range of cellular locations and potential functions, it must be tightly regulated so that it is only active when and where it is required in the cell. Although few precise details are known about how myosin VI is regulated, at least four potential regulatory mechanisms exist (which may act co-operatively; figure 8).

(a) Myosin VI isoforms

Myosin VI has two unique inserts in its tail domain (figure 8a) and can thus exist in four alternatively spliced isoforms (with a large, small, both or no insert in its tail domain) that exhibit distinct tissue distributions (Buss *et al.* 2001). For example, the isoform with the large insert is required for targeting to the apical surface in polarized cells, whereas the isoforms with the small or no insert are involved in membrane transport in non-polarized cells. Thus the single myosin VI gene gives rise to multiple motors with apparently distinct cargo-binding specificities and cellular functions.

(b) Calcium and calmodulin

It has recently been shown that the unique 53 amino acid insert just after the motor domain in myosin VI unexpectedly binds a calmodulin molecule (Bahloul *et al.* 2004). This insert appears to be the reverse gear responsible for movement towards the minus end of actin filaments (Wells *et al.* 1999). So with the conventional single IQ motif (light chain binding domain), myosin VI binds two calmodulins but only the calmodulin bound to the IQ motif is believed to act as a calcium sensor (figure 8a). Although previous kinetic studies indicated that Ca^{2+} had no major effect on the kinetic properties of myosin VI (Yoshimura *et al.* 2001), the most recent data (Morris *et al.* 2003) indicates that increases in Ca^{2+} concentration (more than 10 μ M) lead to a reduction in the rate of ADP release from actomyosin VI and to a slowing down in the *in vitro* actin filament sliding velocity of the myosin VI artificial dimer constructs. Unlike molluscan myosin II, where calcium binding to the light chains operates as a complete regulatory switch, calcium does not function as an 'on/off' switch for myosin VI motility but rather appears to have a modulatory role.

(c) Phosphorylation

A potential phosphorylation site has been identified in myosin VI at T⁴⁰⁶ in the 'cardiomyopathy' loop at the actin binding interface in the motor domain (figure 8a). This site is equivalent to the serine residue first identified in *Acanthamoeba* myosin I, which must be phosphorylated for complete actomyosin I motility (Brzeska *et al.* 1997). The kinase involved is thought to be a PAK, a rac- and cdc42-activated kinase closely related to the *Acanthamoeba* heavy chain kinase. There is, however, some dispute regarding the effects of phosphorylation at this site on myosin VI activity *in vitro*. Yoshimura *et al.* (2001) suggested, based on their *in vitro* kinetic data, that phosphorylation at T⁴⁰⁶ serves as an on/off switch for myosin VI motility, whereas more recent data from Morris *et al.* (2003) concluded that phosphorylation at this site had no effect on either the velocity of actin filament sliding or the maximal actin-activated ATPase activity of myosin VI. We observed that mutating T⁴⁰⁶ in GFP-myosin VI to either an A or E residue to mimic the non-phosphorylated or phosphorylated state had no obvious dramatic effect on GFP-myosin VI activity *in vivo*. The effect of phosphorylation at T⁴⁰⁶ in a strategic loop in the actin binding interface is therefore intriguing; maybe in the cell it plays a rather subtle role, fine-tuning the interactions of myosin VI with the actin cytoskeleton? We have identified additional phosphorylation sites in the tail domain of myosin VI, which appear to

Figure 7. A possible model to explain the large working stroke of our myosin VI. We propose that the size of the working stroke of myosin VI is a combination of lever arm length, lever arm rotation and orientation with regard to the actin filament. A lever arm length of 10 nm could be generated by the single IQ motif, the 53 amino acid insert now known also to bind calmodulin and a contribution from the first part of the helical tail. Cryo-EM images of myosin VI bound to an actin filament (Wells *et al.* 1999) showed that in ADP (at the end of the powerstroke and release of P_i) the lever arm points at an angle of 65° towards the minus end of the actin filament, and upon ADP release, it changes to the rigour orientation of 85° towards the minus end. We calculate that a pre-powerstroke orientation of the 10 nm lever arm of -53° and then a 138° rotation of this lever arm between this pre-powerstroke and ADP (65°) orientations could generate the first substep of 17 nm observed in the optical trap measurements (Lister *et al.* 2004). The movement of the lever arm associated with ADP release and a change from the 65° ADP to 85° rigour orientations account for the second small substep of 0.9 nm.

regulate the binding of at least one of myosin VI's binding partners (R. Roberts and G. Spudich, unpublished results). At present we know very little about the kinases, phosphatases, signalling pathways and actual phosphorylation sites that might be involved in regulating myosin VI activity in the cell. Are they different in the different cellular pathways such as occur at the plasma membrane or at the Golgi complex?

(d) *Conformational changes in myosin VI?*

The tail region of myosin VI can exist as a stable monomer and can be induced to form a dimer by inclusion of a leucine zipper motif or a short region of myosin II coiled-coil domain (Rock *et al.* 2001; Nishikawa *et al.* 2002). Do monomer and dimer forms of myosin VI exist in cells and, if so, how is this interchange regulated (figure 8b)? Secondary structure analyses on the tail (Lister *et al.* 2004) predict that the first part contains a helical region interrupted by hinges and loops (possibly forming a helix-loop-helix structure) followed by a central domain with alternate

repeats of four negatively and four positively charged amino acids that could form a salt bridge-stabilized helix with considerable flexibility (figure 8). The C-terminal end of the tail (the globular tail domain) has no obvious predicted structure but contains the binding sites for the large number of myosin VI binding partners that have so far been identified. How is binding to these sites on the globular tail regulated? One possible model that might answer the above intriguing questions is as follows: in the monomer (in the off state) the tail is folded so that the binding sites in the globular tail and the region(s) involved in dimerization are blocked or hidden. Upon activation (ATP binding, an increase in $[Ca^{2+}]$ and/or phosphorylation or non-phosphorylation or other signal?), the tail unfolds to first allow interaction with its binding partners or lipid membranes and then dimerization may or may not ensue (depending on which type of binding partner is bound). Simultaneously the motor domain may also be switched on and the myosin VI can undergo its specific task. The inserts in the tail might be involved and could play a part in the

Figure 8. (a) Possible mechanisms for regulating myosin VI. P, phosphorylation sites in the motor domain (near the actin binding interface) and in the globular tail domain near the cargo-binding sites; Ca^{2+} , calcium binding to the calmodulin present in the neck region; 'large' and 'small' inserts are present in the globular tail which give rise to four differentially spliced variants with tissue-specific expression; flexible region?, a highly charged domain in the centre of the helical tail region which may be flexible and control the 'conformation' of the tail. (b) Is myosin VI a monomer? Does it function as a non-processive monomer when (i) it tethers a vesicle or (ii) when it binds to membranes to maintain their shape and rigidity, for example to maintain the organization of the Golgi complex and plasma membrane, or does it depend on which binding partners it binds? Can myosin VI also be a processive dimer? When (iii) it binds to lipid vesicles or (iv) to certain binding partners, could it dimerize for transport around the cell?

type of conformational change undergone. The recently identified phosphorylation sites in the tail (R. Roberts, unpublished results) may be one of the regulatory signals.

Although there is little direct evidence for this model for myosin VI at present, such a scheme is believed to occur in non-muscle myosin II at least *in vitro* (Craig *et al.* 1983). When the regulatory light chain in this myosin II is dephosphorylated then the tail folds so that the myosin is in a compact inactive state (even the ATPase and actin binding activities in the motor domain are switched off). When the light chain is phosphorylated by the specific Ca^{2+} and calmodulin-activated myosin light chain kinase then the tail unfolds and assembles to form bipolar filaments that interact with and move actin filaments *in vitro* (Craig *et al.* 1983). Recent evidence (Wang *et al.* 2004) indicates that myosin V also folds into a compact inactive conformation in the absence of Ca^{2+} and unfolds into an extended conformation in the presence of Ca^{2+} or at high ionic strength. EM images show that, in the absence of Ca^{2+} , the myosin V heads and tail are folded together to form a compact triangular shape.

Can myosin VI also exist in such an inactive folded conformation? The single myosin VI images seen in the EM confirm that the tail domain appears to be extremely flexible and able to undergo large changes in conformation on addition of ATP. The image of myosin VI with a sharply bent tail (figure 6c) in the absence of nucleotide would support the idea that myosin VI might adopt a folded

conformation. Future biophysical and structural analysis of myosin VI molecules in different states, for example, with nucleotide and nucleotide analogues, with or without phosphorylation and/or Ca^{2+} , and in the presence of different binding partners and/or defined lipid membranes, may provide confirmatory evidence of whether myosin VI can fold into a compact inactive conformation and whether it exists as both a monomer and dimer *in vivo*.

13. PERSPECTIVES

It is now 12 years since myosin VI was first identified in *Drosophila* (Kellerman & Miller 1992) and it has subsequently been identified in most higher eukaryotic animals (Berg *et al.* 2001). However, it is absent in *Dictyostelium*, yeast and plants. Because myosin VI moves towards the minus end of actin filaments unlike almost all the other myosins so far studied in detail, what minus-end-directed myosin takes its place in these organisms? Or are minus-end-directed motors not required in these organisms? The observation that the absence of myosin VI in mice is not lethal implies that another minus-end-directed motor exists and that this can replace the missing myosin VI in *sv* mice. Initial evidence suggested that myosin IX could be a candidate because expressed myosin IX (without the tail domain) was observed to be a minus-end-directed motor (Inoue *et al.* 2002), but subsequent studies with whole native myosin IX indicated that it moved towards the plus end of actin filaments (O'Connell & Mooseker 2003).

The directionality of myosin IX movement therefore needs to be resolved. The fascinating demonstration (Tsiavaliaris *et al.* 2004) that molecular engineering can be used to convert a plus-end-directed myosin into a minus-end-directed motor by inserting a specific structural domain into the neck region raises the intriguing possibility that nature could potentially use a similar mechanism in the cell?

Dramatic progress in the myosin field as exemplified by the work on the class V myosins has been achieved in the last few years, driven by the concerted employment of a wide variety of new and old technologies, such as single molecule 'optical tweezer' and fluorescent probe (total internal reflection fluorescence (TIRF) and fluorescence imaging with one nanometre accuracy (FIONA)) techniques, atomic force microscopy, rapid kinetics, live cell microscopy, protein engineering and 'knock down' technology, together with structural analysis (X-ray crystallography and electron microscopy) (reviewed by Vale 2003). Exciting new developments in the myosin VI field using a similar combination of techniques are now starting to emerge, but unlike the story with myosin V, they have so far raised more questions than answers. For example, how can myosin VI with such a short lever arm achieve such a large step size? Is myosin VI a non-processive monomer or processive dimer in the cell? Or can it exist as both species depending on different intracellular requirements, i.e. membrane stabilisation versus membrane transport (figure 8b)? If so, are the monomer and dimer states of myosin VI exchangeable and how could this switch be regulated in the cell? What roles do the different binding partners in the different cellular localizations play? Answers to these and many other questions will surely emerge very soon, especially with information on the structures of the motor domain, neck region and tail domains, together with the novel tools and reagents being developed to probe the behaviour of single myosin VI molecules *in vitro* and *in vivo*.

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GLOSSARY

- ADP: adenosine diphosphate
 AMPA: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
 ATP: adenosine triphosphate
 BAL: B-aggressive lymphoma
 CCP: clathrin-coated pit
 Dab2: disabled-2
 EM: electron microscope
 GFP: green fluorescent protein
 GIPC: GLUT1 C-terminal binding protein
 GST: glutathione-S-transferase
 IQ: isoleucine–glutamine
 LDLR: low density lipoprotein receptor
 MW: molecular weight
 NPxY: asparagine, proline, any amino acid, tyrosine
 PAK: p21-activated kinase
 P_i: phosphate
 SAP97: synapse-associated protein 97
 sv: *Snell's waltzer*