Minireview

Myosin VI, a new force in clathrin mediated endocytosis

Folma Buss[a,*], J. Paul Luzio[a], John Kendrick-Jones[b]

[a] *Department of Clinical Biochemistry and Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK*
[b] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

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Abstract The integrity of the actin cytoskeleton and associated motor proteins are essential for the efficient functioning of clathrin mediated endocytosis at least in polarised cells. Myosin VI, the only motor protein so far identified that moves towards the minus end of actin filaments, is the first motor protein to be shown to associate with clathrin coated pits/vesicles at the plasma membrane and to modulate clathrin mediated endocytosis. Recent kinetic studies suggest that myosin VI may move processively along actin filaments providing clues about its functions in the cell. The possible role(s) of myosin VI in the sequential steps involved in receptor mediated endocytosis are discussed.

Key words: Myosin; Endocytosis; Clathrin; Polarised cell

1. Introduction

In eukaryotic cells endocytosis is essential for the uptake of many nutrients, for defence against invading microorganisms and for the regulated uptake of cell surface receptors, thus linking endocytosis to cell signalling [1,2]. Following internalisation the endocytosed material is transferred to early endosomes from where it is either recycled back to the cell surface or transported further into the cell, where it can be found in late endosomes or lysosomes. The site of endocytosis is the plasma membrane, which is directly linked to the underlying actin network in the cell cortex. Genetic evidence in yeast links the organisation of the actin cytoskeleton to both receptor mediated and fluid phase endocytosis [3,4]. In higher eukaryotic cells the link between endocytosis and the actin cytoskeleton is less certain and the evidence conflicting, being dependent on the cell type and the assay used [5,6]. However, since an increasing number of proteins associated with the endocytic machinery also interact with the actin cytoskeleton a link is strongly suggested (reviewed in [7]). The filamentous actin network could either provide a structural framework for the spatial organisation of the endocytic machinery and/or serve a more indirect function by providing the tracks for the movement of myosin motor proteins involved in force generation and vesicle movement.

2. Myosins

The myosins are a diverse superfamily of actin activated mechanoenzymes that use the energy from ATP hydrolysis to generate force and movement along actin filaments. Each myosin can be divided into two functional domains: (1) an N-terminal motor domain, which is comprised of a catalytic domain that binds actin and ATP, a 'converter' region and a regulatory neck region ('lever arm') that binds light chains or calmodulin and (2) a C-terminal tail domain, which anchors the myosin and may carry cargo [8]. The myosin superfamily comprises at least 18 different classes and so far only myosins in classes I, V, VI and VII have been directly implicated in membrane traffic [9].

3. Myosin VI, a motor protein with unique properties

In this review we focus on myosin VI and discuss its possible role(s) in receptor mediated endocytosis. Myosin VI is ubiquitously expressed in higher eukaryotic tissues, but is absent in *Dictyostelium* and yeast. It has been shown to be important for hearing in mouse and human [10,11] and for embryogenesis and spermatogenesis in *Drosophila* [12,13]. Myosin VI is a dimeric molecule with two motor domains, a short region of coiled coil and two globular tails. We have shown that myosin VI is phosphorylated in vivo and that it can be phosphorylated in vitro in the motor domain by a p21 activated kinase (PAK) [14]. Although the phosphorylation site has not yet been directly identified, considerable circumstantial evidence indicates it is T_{406} in agreement with the TEDS rule (consensus sequence position in the motor domain present in nearly all myosins which is either a threonine (T), glutamate (E), aspartate (D) or serine (S)) proposed by [15]. Recent kinetic data [16] measuring the rates of the steps in the ATPase cycle of myosin VI have shown that a mutant ($T_{406}E$) mimicking phosphorylation at the T_{406} site has an accelerated rate of P_i release in the presence of actin. Yoshimura et al. [17] however find that phosphorylation of myosin VI at T_{406} is not required for actin activated ATPase activity but is essential for generating actin filament sliding. How phosphorylation activates the motile activity of myosin VI is not known. Both groups [16,17] agree that myosin VI has a high affinity for ADP in the presence of MgATP and remains strongly attached to actin during most of its ATPase cycle. Unlike all the other myosins that have so far been studied, which move towards the plus end of actin filaments, myosin VI is unique in moving towards the minus ends of actin filaments

*Corresponding author. Fax: (44)-1223-762740.
E-mail address: fb1@mole.bio.cam.ac.uk (F. Buss).

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[18]. Myosin VI has a unique 53 amino acid insert in the converter region of the catalytic domain between the motor domain and the lever arm, which was thought to be at least partly responsible for the movement in the reverse direction. However it has recently been demonstrated [19] using chimaeric myosins composed of motor domains and lever arm domains from myosins that move in opposite directions on actin, that it appears to be the core motor domain that determines the direction of myosin movement. The minus end directionality would support a role of myosin VI as an endocytic motor protein, since inside cells the actin filaments which are present in filopodia, lamellipodia, microvilli, stereocilia and in the actin cortical network have their plus ends inserted into or pointed towards the plasma membrane and their minus ends pointing into the cell. Myosin VI moving towards the minus ends of actin filaments would thus move endocytic cargo vesicles into the cell (see Fig. 3).

4. Myosin VI in polarised cells

Myosin VI is expressed as three different splice variants containing either a large, a small or no insert in the tail domain, as first described in *Drosophila* [20] and more recently in fish [21]. In mammalian cells these splice isoforms display a tissue specific distribution with the splice variant containing the large insert specifically found in tissues containing polarised cells with microvilli at their apical surface [22]. In polarised cells from the intestinal brush border, myosin VI is concentrated in the terminal web [22,23] with small amounts in the microvilli themselves. Myosin VI present at the apical domain is localised to clathrin coated pits/vesicles. In non-polarised cells the myosin VI isoforms without the large insert are expressed and these colocalise to a much lesser extent with clathrin coated pits/vesicles [22]. Overexpression of GFP tagged myosin VI containing the large insert, cloned from a polarised tissue, in unpolarised fibroblastic cells results in co-localisation with clathrin coated pits/vesicles (see Fig. 1 and [22] for more detail). The association of myosin VI with the endocytic machinery in polarised cells appears to be one of the key functions of myosin VI. Absence of myosin VI in *Snell's Waltzer* mice (myosin VI knock out) results in defects which are most apparent in polarised cells; for example the lack of myosin VI leads to degeneration of the stereocilia, which resemble highly specialised microvilli at the apical domain of the hair cells of the inner ear, and this results in a loss of hearing [24]. In addition, in these mutant mice the microvilli on intestinal brush border cells are shorter when compared to WT mice ([24], Buss and Stewart, unpublished observation). Since the *Snell's Waltzer* mice survive there must be motors and possibly other proteins, which are able to at least partially compensate for the loss of myosin VI in most cells apart from those in the inner ear.

5. What precise role(s) does myosin VI have in receptor mediated endocytosis?

The clear association of myosin VI with clathrin coated pits/vesicles in polarised cells, raises the question at which step in the formation and the transport of a clathrin coated pit or vesicle is myosin VI involved? Is myosin VI required for (1) the spatial organisation of the endocytic machinery in the plasma membrane; (2) sequestering of receptors and coated pit formation; (3) invagination of the plasma membrane; (4) pinching off newly formed vesicles or (5) the transport of vesicles away from the plasma membrane into the cell?

The specific localisation of the myosin VI isoform with the large tail insert to endocytic clathrin coated vesicles concentrated at the apical domain of polarised cells compared to non-polarised fibroblastic cells may provide us with a clue as to which step in the pathway myosin VI is involved. As mentioned above actin may not be obligatory for receptor mediated endocytosis per se in all cell types. For example depolymerisation of the actin cytoskeleton using drugs like cytochalasin D and latrunculin A, which cause F-actin filament disassembly, does not appear to inhibit receptor mediated endocytosis in non-polarised, adherent A431 or COS-7 cells [5]. In contrast, in polarised cells containing apical microvilli with their highly specialised actin cytoskeleton and plasma membrane organisation, as for example in human intestinal brush border Caco-2 cells or MDCK cells (Madin–Darby canine kidney cells), the requirement for actin in clathrin coated vesicle uptake from the apical domain is unambiguous. Treating these cells with cytochalasin D blocks clathrin coated vesicle uptake specifically at the apical domain and leads to an increase in the number of coated pits at the apical surface (Fig. 2a), indicating that actin may be important for pinching

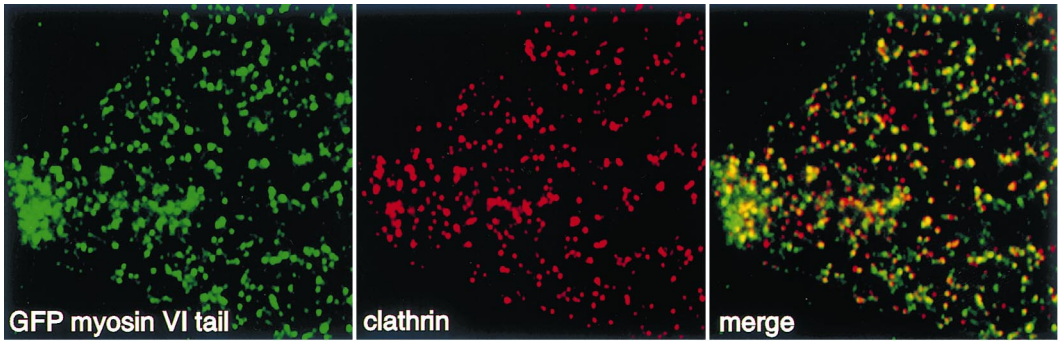

Fig. 1. Localisation of myosin VI in clathrin coated vesicles. Normal rat fibroblasts (NRK cells) were transfected with GFP tagged myosin VI tail and the transfected cells were labelled with antibodies to clathrin as described in Buss et al. [22]. The yellow punctate pattern in the merged image clearly indicates partial colocalisation of myosin VI tail (green) and clathrin (red). Clathrin coated vesicles without myosin VI either represent a population of coated vesicles containing AP1 or AP3 from other membrane traffic pathways or it could indicate that myosin VI comes off a clathrin coated vesicle before uncoating takes place. Furthermore using antibodies to myosin VI not only clathrin coated vesicles are stained, but also a number of other vesicular structures, which have not yet been identified, indicating that myosin VI might act as a more universal motor.

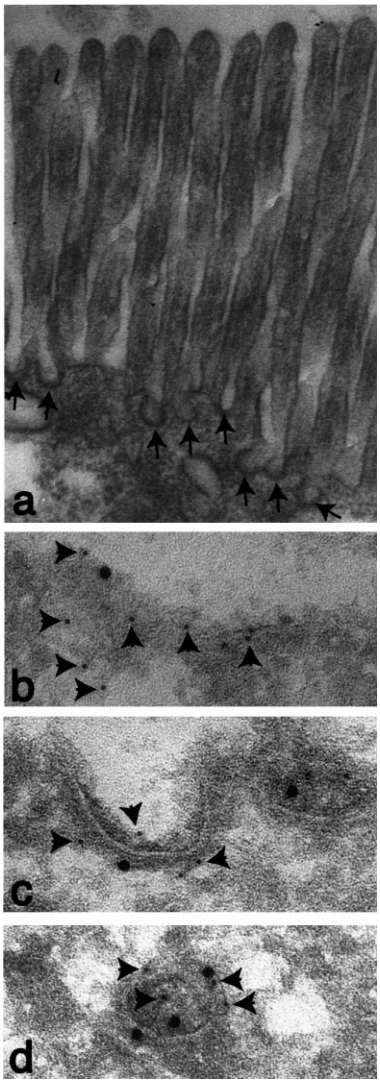

Fig. 2. Electron micrograph (a) of a section of the apical domain of polarised human intestinal brush border cells (Caco-2 cells) after cytochalasin D treatment [27]. Depolymerisation of actin filaments clearly increases the number of clathrin coated pits at the base of microvilli. Gold label indicates molecules of ricin binding to the microvillar surface. b–d: Immuno EM localisation of myosin VI (5 nm gold) labelled with arrowheads and clathrin (15 nm gold) in NRK cells [22]. In b myosin VI is associated with an early stage of clathrin coated pit formation, whereas in c it can be seen in a deeply invaginated pit and in d myosin VI is associated with a clathrin coated vesicle in the cytosol.

off vesicles from the plasma membrane [25–27]. We therefore believe that actin, in association with motor proteins like myosin VI, is needed for receptor mediated endocytosis in certain cells under specific physiological conditions or in cells with highly specialised plasma membrane domains.

6. Sequential steps in endocytosis

Clathrin mediated uptake at the plasma membrane can be divided into a number of steps (see Fig. 3) and in this review we will discuss the evidence for myosin VI involvement in each. At the EM level myosin VI can be found at a very early stage of clathrin coated pit formation, at the step where coated pits are deeply invaginated and it is also present in clathrin coated vesicles, which appear to have pinched off from the plasma membrane (Fig. 2b).

6.1. Spatial organisation of the endocytic machinery

At first glance clathrin coated pits in non-polarised cells seem to be uniformly distributed over the surface of the whole cell. However, using GFP tagged clathrin in live cells it has been revealed that every single coated pit shows a very restricted movement in the plane of the plasma membrane [28]. Applying low concentrations of latrunculin A increased the mobility of clathrin coated pits indicating that actin provides a framework for the organisation of clathrin coated pits at the plasma membrane. The endocytic machinery is organised in 'hot spots' by binding to the underlying cortical actin network generating preferential sites of coated pit formation. At the apical domain of polarised cells, localisation of clathrin coated pits is even more restricted to the area of the plasma

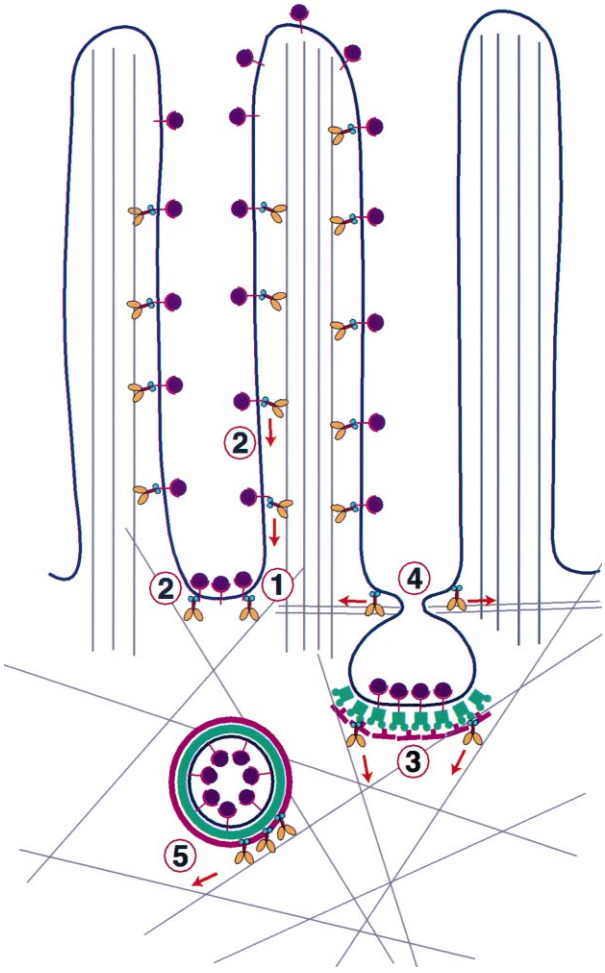

Fig. 3. Cartoon illustrating the possible involvement of myosin VI (arrows indicate direction of myosin VI movement) in sequential steps of endocytosis in polarised cells containing microvilli at their apical domain: (1) spatial organisation of the endocytic machinery at the base of microvilli; (2) movement of receptors down to the base of a microvillus for sequestering and coated pit formation; (3) invagination of the plasma membrane; (4) vesicle scission with myosin VI attached to the plasma membrane moving towards the minus end of actin filaments and thereby driving the filament to constrict the neck; (5) transport of clathrin coated vesicles away from the plasma membrane into the cell.

membrane at the base between adjacent microvilli. An actin associated motor protein like myosin VI could provide a flexible linker between the clathrin coated pit and the actin cytoskeleton, being able to adjust the position of the clathrin coated pits dependent on the physiological state of the cell.

6.2. Sequestering of receptors and coated pit formation

Work by Santini and Keen [29] has suggested that receptors migrate into a pre-existing coated pit rather than activated receptors recruiting coat components from the cytosol to form a pit. In non-polarised cells, receptors probably have less distance to migrate into the next coated pit than at the apical surface of polarised cells, where a receptor has to travel down a microvillus to enter a coated pit at its base. In Caco-2 cells it has been shown that ricin (a ligand which binds to galactosylated membrane proteins and lipids and is endocytosed via clathrin coated vesicles) requires actin filaments to move along the surface of microvilli [27]. Since the plus ends of actin filaments are at the tips of microvilli, the movement of plasma membrane components down the microvillar surface requires a minus end directed reverse motor like myosin VI. This myosin has been localised to brush border microvilli [23].

6.3. Invagination of the plasma membrane

At present there are several candidate mechanisms for invagination of the plasma membrane including an asymmetric lipid composition and a role for endophilin (for review see [30]). Myosin VI bound to assembled coat components [22] would be able to move along actin filaments into the cell, pulling the plasma membrane inwards and thereby increasing the curvature.

6.4. Vesicle scission

Dynamin has been implicated in vesicle scission although its precise function remains controversial [31]. It has been shown to bind to several actin binding proteins including profilin (a G-actin binding protein) [32] and syndapin, which in turn binds to N-WASP [33,34]. N-WASP is the neuronal isoform of the Wiskott–Aldrich syndrome protein, which regulates actin polymerisation via the Arp2/3 complex. Thus both G-actin binding proteins, profilin and syndapin, could potentially link dynamin to sites of new actin polymerisation assembled at the neck of a forming clathrin coated vesicle. Myosin VI could work in this newly polymerised actin network at the site of vesicle fission, by moving actin filaments towards the invaginated plasma membrane and thereby pushing the membranes of the vesicle neck together. Actin itself has been reported in several examples to be involved in pinching off clathrin coated vesicles. In Caco-2 and MDCK cells after cytochalasin D treatment there is an increase in the number of coated pits with long necks at the apical domain suggesting that vesicle fission is impaired [25,27].

6.5. Clathrin coated vesicle dynamics and transport

Using GFP tagged clathrin light chains in live cells, Gaidarov et al. [28] have shown that clathrin coated vesicles move and display a lifetime of a few seconds before uncoating. Our unpublished data with GFP tagged myosin VI in live cells show similar vesicle movement, suggesting that myosin VI is indeed able to move vesicles along actin tracks.

In Fig. 3, steps 1–5 of clathrin vesicle formation are shown as a cartoon, highlighting the possible function of myosin VI in each step.

7. What kind of motor is myosin VI? Is it processive or non-processive?

Recent work by a number of groups [16,17,19] probing the kinetic mechanism of myosin VI activity provide us with further clues to the potential role(s) of this myosin in the cell or at least may exclude certain functions. They demonstrate that ADP release was the rate limiting step in the ATPase cycle and that the myosin VI ADP state bound strongly to actin. Furthermore their kinetic data suggested that myosin VI could be a processive, dimeric (two motor domain) motor with at least one motor domain always attached to actin; in other words it has a high duty ratio (it spends a significant proportion of its ATPase cycle strongly bound to actin) and is a motor ideally adapted for maintaining tension. De La Cruz et al. [16] propose an 'alternating site' model for the stepping and processivity of the dimeric myosin VI along actin filaments which may allow us to further define the role of this motor in endocytic pathways; for example myosin VI by exerting tension could anchor the endocytic machinery to the underlying actin cytoskeleton or pull membrane into the cell to form a clathrin coated pit or transport endocytic vesicles processively along actin filaments into the cell. Myosin V has similar properties to myosin VI [16] and has been shown to move processively along actin filaments but in the opposite direction [38,39]. Myosin V has a long lever arm with six IQ (calmodulin binding) repeats and can achieve a step size of 36 nm 'walking' along an actin filament (equivalent to the helical repeat of the actin filament) whereas myosin VI has a short lever arm with only a single IQ repeat. So if myosin VI is processive and needs a large step size to move vesicles in a straight path along an actin filament then it would require that either the coiled coil domain of the tail unfolds possibly involving the region of charged repeats observed in the middle of this domain [14] and/or the flexibility of the insert between the motor domain and lever arm changes or some other still to be identified mechanism acts to achieve such a step. Future studies promise to yield exciting results.

8. Myosin VI as an endocytic motor in differentiation

If myosin VI works as an endocytic motor, how can we explain its key role in hearing, embryo development and spermatogenesis? In *Snell's Waltzer* mice, which are missing myosin VI, the major phenotype affects the sensory hair cells of the inner ear. At birth the rows of microvilli in the hair cells which form the stereocilia have a normal appearance but 3 days after birth as the stereocilia elongate and mature, they start fusing together. In wild type mice this phase of stereocilia maturation is accompanied by extensive membrane recycling at the apical domain of these hair cells and clathrin coated pits can easily be visualised at the base of immature stereocilia [35]. Since myosin VI has been shown to specifically associate with clathrin coated pits/vesicles at the apical domain of polarised cells, it is likely that myosin VI plays a vital role in endocytosis at the apical domain during hair cell maturation. In *Drosophila*, myosin VI is required at various developmental stages including imaginal disc morphogenesis, follicle cell epithelial morphogenesis [36], formation of mem-

brane invagination in the syncytial blastoderm [12] and sperm individualisation during spermatogenesis [13]. The latter process involves the formation of a complex machinery bringing together cytoskeleton and membrane remodelling. The only proteins identified so far as being crucial in this process are myosin VI [13], actin and the coat protein clathrin [37]. This evidence indicates that endocytosis and membrane recycling form an important step during sperm individualisation and provide further support for the proposal that myosin VI works as an endocytic motor.

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