Effects of loss of myosin VI no-insert isoform on clathrin-mediated endocytosis of plasma-membrane receptors

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Myosin VI is an unconventional myosin as it can move towards the minus-end of actin, and it is involved in different cellular functions, such as endocytosis, exocytosis and cytokinesis.1,2 These different functions are related to myosin VI binding with different partners, including Dab2, GIPC, T6BP, LMTK2 and the lipid phosphatidylinositol 4,5-bisphosphate (PtdInsP2).3-7 Myosin VI localizes on clathrin-coated structures, and its role in clathrin-mediated endocytosis is tightly connected to its interactions with the binding partner Dab2 and with PtdInsP2.7

Fibroblasts from Snell’s waltzer mice (myosin VI knock-out mice) have a severe defect in endocytosis, and in particular in clathrin-coated vesicle internalization.8 Moreover, loss of myosin VI induces re-localization of plasma-membrane receptors, such as transferrin and epidermal growth factor receptors, from clathrin-coated structures to caveolae. Under these conditions, the clathrin adaptor AP-2 also follows the receptors into the caveolae.9

Endocytosis is an important mechanism that is responsible for exchanges between the cell and its external environment, and in particular for the regulation of signal transduction, immune surveillance, antigen presentation, cell-cell communication and cellular homeostasis.10

Clathrin-mediated endocytosis is by far the best-characterized endocytic pathway in eukaryotic cells. Plasma membrane receptors recruit adaptor proteins to the plasma membrane and then clathrin molecules. When the clathrin-coated pit is loaded with receptors, it can invaginate and “pinch off” the plasma membrane, to form a clathrin-coated vesicle.3 These plasma-membrane receptors are recognized by the clathrin adaptor AP-2 through the short amino-acid motifs in its cytoplasmic tail, including YXXφ (where φ represent one of the amino acids F, I, L, M or V) and its acidic di-leucine sequence.10,11 This AP-2 binding to the plasma membrane also depends on interactions with the lipid phosphatidylinositol 4,5-bisphosphate (PtdInsP2), which is weaker in the presence of the di-leucine motif.10,11

Other endocytic pathways co-exist in the cell, and sometimes these can compensate for loss of plasma-membrane receptor internalization when clathrin-mediated endocytosis is defective.12-14 Among these clathrin-independent pathways, the caveolae are the best characterised.15 I recently demonstrated that when myosin VI is lost from the cell, plasma-membrane receptors, such as the transferrin receptor (TfR), are also endocytosed via caveolae.8 The major goal of my study was to show that the myosin VI isoform without the insert (the no-insert isoform; NoI) has an important role in clathrin-mediated endocytosis. A similar function was previously described for the myosin VI isoform that contains the large insert (LI) in polarized cells.2

For this recent study, I used non-polarized cells that do not express myosin VI LI: fibroblasts and HeLa cells. Loss of myosin VI NoI thus led to a defect in clathrin-mediated endocytosis, with a strong reduction in vesicle delivery. However, plasma-membrane receptors expressing the YXXφ motif, as for the TfR, were internalized normally, with comparable speed.
and timing to the control cells. The strong reduction in clathrin-coated vesicle assembly and the normal rate of internalization of the TfR in this absence of myosin VI could be explained only by the upregulation of an alternative endocytic pathway that can compensate for this defect in clathrin-mediated endocytosis.

Electron microscopy analysis showed that in the absence of myosin VI, the TfR localized almost completely on caveolae, and only the use of caveolae-blocking agents (i.e., filipin and C8-LacCer) or caveolin-1 siRNA knock-down blocked TfR internalization in these cells. These data not only demonstrate that when myosin VI is lost, the TfR relocates on caveolae, but also that caveolae represent an alternative endocytic pathway that can be upregulated to compensate for a defect in another pathway.

This switch from clathrin to caveolae is probably possible only under some conditions, one of which would be where the clathrin adaptor protein AP-2 can follow the receptor and bind to PtdIns(4,5)P2 localized in another endocytic pathway, different from that for clathrin. It is well known that caveolae are enriched in PtdIns(4,5)P2, so this pathway represents a good alternative for receptor internalization. Indeed, in this same study, I also showed that when myosin VI is lost, not only do the receptors localise on caveolae, but AP-2 does also. Moreover, the rate of co-localization for the receptors and the AP-2 adaptor with and without myosin VI is the same, leading to the demonstrate that the TfR and AP-2 move and are internalized together, whatever endocytotic pathway is used.

The role of myosin VI has been discussed in great detail previously, and the common strategy has been to try to connect a function of myosin VI in endocytosis with its localization on clathrin-coated structures. In my recent study, a defect in clathrin-mediated endocytosis when myosin VI is lost was visualized for the first time, and even if the localization of myosin VI NoI in clathrin-coated structures on the plasma membrane was not strong (25%), it was highly specific. Although this relocalization was shown for the prototype receptor for clathrin-mediated endocytosis, the TfR, the strong defect in clathrin-coated vesicle internalization suggested that other receptors that express the YXXØ motif, such as the epidermal growth factor receptor (EGFR), show the same behavior. For this reason, EGFR localization was also analyzed in cells lacking myosin VI. Here again, the EGFR relocalized in caveolae in fibroblasts from Snell’s waltzer mouse and with siRNA knock-down for myosin VI in Hela cells (Fig. 1A–C). Also similar to the TfR, Hela cells with the double siRNA knock-down for myosin VI and caveolin-1 did not internalize EGF when the rate of internalization in cells lacking myosin VI or caveolin-1 alone were comparable with the control (Fig. 1D). These data show that when myosin VI is lost, as with the TfR, the EGFR also re-localizes and is internalized by caveolae. We have also observed similar behavior for a plasma-membrane-receptor chimera (data not shown), the CD8-cation-independent mannose-6-phosphate receptor that also has the YXXØ motif.\(^{18}\)

In summary, I can conclude that myosin VI NoI has an important role in clathrin-mediated endocytosis, and that this role appears to be connected with its binding partner PtdIns(4,5)P2. The myosin VI function here could be to recruit and accumulate this lipid in clathrin-coated structures on the plasma membrane, and thus the loss of myosin VI would create clathrin-coated structures that are poor in PtdIns(4,5)P2. Under this condition, AP-2 can bind to the PtdIns(4,5)P2 that is in another endocytic pathway, such as that for caveolae, and the plasma-membrane receptors expressing the YXXØ motif would re-locate and thus be internalized by this alternative pathway (Fig. 2). Although a myosin VI function in PtdIns(4,5)P2 accumulation in clathrin-coated structures remains to be proven, this represents an intriguing model for further investigation. This possibility of using an alternative pathway to compensate for a defect in the clathrin pathway would also explain why the Snell’s waltzer mice can live without showing any major defects.

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Figure 1. Loss of myosin VI also relocalises the EGFR from clathrin-coated structures to caveolae. (A) Ultrathin cryosection from wild-type (wt/wt) and Snell’s walzer mice (sv/sv) were immunolabelled using a rabbit anti-EGFR antibody (SantaCruz Biotechnology, Santa Cruz, CA, USA) or double-labelled with a rabbit anti-caveolin-1 antibody (BD Biosciences, San José, CA, USA) following by protein-A gold. While in the wt/wt fibroblasts the EGFR is only seen in clathrin-coated structures (a and c), in the sv/sv fibroblasts it is almost completely relocated to non-coated structures (b–d) that are caveolin-1 (cav1) positive. Bar: 300 nm. (B) Quantification as in (A) of morphometric analyses of labelled and unlabelled organelles in 1,000-µm cell profiles. The relative amounts of EGFR in clathrin-coated structures (CCS) and caveolae (Cav). (C) Quantification as in (A) of relative amounts of EGFR present in clathrin-coated structures (CCS) and caveolae (Cav). (D) HeLa cells were either mock-transfected or transfected twice with siRNAs specific for myosin VI (MVI) and/or caveolin-1 (cav-1). The cells were serum-starved for 20 h, and incubated with Alexa-EGF-555 on ice for 1 h, which was internalised for 10 min at 37°C. The EGF left on the plasma membrane was removed with an acid wash, and the samples were analysed with a Zeiss LSM-510 confocal microscope. Time-course analysis to 20 min of random sample fields of 150 x 150 pixels using the Volocity software (Improvision-Perkin Elmer version 5). MVI siRNA KD, siRNA knock-down for myosin VI.
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**Figure 2.** Summary model for the potential role of myosin VI in clathrin-mediated endocytosis. (A) Myosin VI localization to clathrin-coated pits and its binding to PtdInsP2 implies the recruitment and concentration of PtdInsP2 in these clathrin-coated pits on the plasma membrane (a–c). The clathrin-coated pits enriched in PtdInsP2 can recruit the clathrin adaptor protein AP-2 and plasma-membrane receptors, and in particular those that express the YXXϕ motif (d). (B) In non-polarised cells lacking myosin VI, AP-2 is recruited into caveolae together with plasma-membrane receptors expressing the YXXϕ motif, thus using this alternative pathway to compensate for the defect in clathrin-mediated endocytosis (a and b).