Loss of myosin VI reduces secretion and the size of the Golgi in fibroblasts from Snell's waltzer mice

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Golgi morphology and function are dependent on an intact microtubule and actin cytoskeleton. Myosin VI, an unusual actin-based motor protein moving towards the minus ends of actin filaments, has been localized to the Golgi complex at the light and electron microscopic level. Myosin VI is present in purified Golgi membranes as a peripheral membrane protein, targeted by its globular tail domain. To investigate the function of myosin VI at the Golgi complex, immortal fibroblastic cell lines of Snell's waltzer mice lacking myosin VI were established. In these cell lines, where myosin VI is absent, the Golgi complex is reduced in size by ~40% compared with wild-type cells. Furthermore, protein secretion of a reporter protein from Snell's waltzer cells is also reduced by 40% compared with wild-type cells. Rescue experiments showed that fully functional myosin VI was able to restore Golgi complex morphology and protein secretion in Snell's waltzer cells to the same level as that observed in wild-type cells.

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Introduction

Maintaining the morphology and intracellular positioning of the Golgi complex is vital for the key functions of this organelle in the modification, sorting and transfer of membrane and luminal proteins from the site of synthesis in the endoplasmic reticulum (ER) to lysosomes, secretory vesicles and the cell surface (reviewed by Traub and Kornfeld, 1997). In the cell, the Golgi complex is arranged as a stack of flattened membrane-bound cisternae in the region around the centrosome, the major organizing centre for cytoplasmic microtubules. It is known that there is an intimate relationship between the Golgi complex and the microtubule-based cytoskeleton, and this association plays an important role in Golgi structure and function (reviewed by Lippincott-Schwartz *et al.*, 1998).

In contrast, less is known about the requirement for actin filaments and their associated motor proteins, the myosins, for intracellular Golgi localization, morphology

and membrane transport to and from the Golgi complex (Sellers *et al.*, 2000). Disruption of actin filaments using drugs such as cytochalasin D and *Clostridium botulinum* toxin C2 (Valderrama *et al.*, 1998), or after Ras-induced transformation (Babia *et al.*, 1999), results in the collapse of the reticulate Golgi structure into a tight cluster of membranes around the centrosome. Furthermore, actin filaments have also been implicated in retrograde transport from the Golgi complex to the ER (Valderrama *et al.*, 2001) and in the formation of COPI-coated buds and vesicles (Valderrama *et al.*, 2000). The formation of actin filaments is tightly associated with the Golgi complex, but the precise role of the actin cytoskeleton in Golgi function remains to be established.

The ADP-ribosylation factor ARF 1 is required for assembly of actin on Golgi membranes in addition to its function in the assembly of coat proteins in membrane traffic pathways (Fucini *et al.*, 2000). ARF 1 activation leads to polymerization of two distinct pools of actin onto Golgi membranes (Fucini *et al.*, 2000). Actin recruitment to the Golgi complex also requires cdc42, a Rho family GTPase, that is an important regulator of actin dynamics possibly through activation of the ARP 2/3 complex (Fucini *et al.*, 2002). Cdc42 has also been shown to bind directly to the γ -COP subunit of the COPI coat (Wu *et al.*, 2000). Both forms of cdc42 (activated and nucleotide free) had similar effects on exocytic traffic in polarized cells, inhibiting the transport of basolateral proteins and stimulating the transport of apical proteins, reflecting a possible modulation of the actin cytoskeleton (Kroschewski *et al.*, 1999; Musch *et al.*, 2001).

Various members of the myosin superfamily, namely myosin I, myosin II and myosin VI, are known to be present at the Golgi complex. Myosin I is believed to be involved in translocating vesicles from the Golgi complex to the apical plasma membrane in polarized epithelial cells (Fath and Burgess, 1993, 1994). The role of myosin II in the Golgi complex is unclear. Myosin II was localized at the Golgi complex using a monoclonal antibody (Ikonen *et al.*, 1997). This antibody was later shown to cross-react with coatamer also present on Golgi membranes (Simon *et al.*, 1998). Nevertheless, it has been shown (Musch *et al.*, 1997) that myosin II is involved in the assembly of basolateral transport vesicles carrying vesicular stomatitis virus G protein (VSVG) from the *trans*-Golgi network (TGN) of polarized Madin–Darby canine kidney (MDCK) cells. However, there are conflicting reports as to whether myosin II (Ikonen *et al.*, 1997) is an essential participant in the vesicle budding reaction or, rather, whether it might play a structural role in organizing the actin filaments which are known to surround the Golgi complex.

Myosin VI, a minus end-directed motor moving towards the pointed end of actin filaments (Wells *et al.*, 1999), is localized intracellularly at the Golgi complex, in clathrin-

coated vesicles, in membrane ruffles and in a cytosolic pool (Buss *et al.*, 1998, 2001). The function of a splice variant of myosin VI, containing a large insert in the tail, has been established as being required for clathrin-mediated endocytosis from the apical surface of polarized cells with microvilli (Buss *et al.*, 2001; Morris *et al.*, 2002). The function of myosin VI at the Golgi complex is not known at present. The gene encoding myosin VI is defective in Snell's waltzer mice (Avraham *et al.*, 1995): these mutant mice have an intragenic deletion in the motor domain of the myosin VI gene, leading to absence of detectable myosin VI protein in homozygotes. The Snell's waltzer mouse mutant displays the typical behaviour associated with inner ear defects: deafness, hyperactivity, head tossing and circling. Myosin VI appears to have an essential function in the stereocilia, which are the modified microvilli on the outer surfaces of the inner ear hair cells. In the Snell's waltzer mutant mice, the general organization of the bundles of stereocilia appears broadly normal at birth, but they become progressively more disorganized and eventually fuse, just a few days after birth; as a result, the mice are deaf (Self *et al.*, 1999). We have used immortal cell lines derived from Snell's waltzer mice to investigate the function of myosin VI in the Golgi complex. We observed a statistically significant size difference between the Golgi complexes in Snell's waltzer cells and wild-type cells, and exocytic rates were significantly reduced in the Snell's waltzer cells. Transfected myosin VI constructs were targeted to their expected intracellular locations in these cells, and we have been able to perform 'rescue' experiments on Golgi complex structure and function in the Snell's waltzer cells using whole, fully functional myosin VI.

Results

Myosin VI is present in the Golgi complex

Using a polyclonal antibody, we localized endogenous myosin VI to the Golgi complex of normal rat kidney (NRK) cells (Buss *et al.*, 1998). We confirmed this localization by indirect immunofluorescence using several different polyclonal and monoclonal antibodies raised against the very C-terminal globular tail domain or the whole tail region. Myosin VI co-localized with the TGN marker, TGN38 (Figure 1). Although there was a clear concentration of myosin VI at the Golgi complex, myosin VI was also present as a cytosolic pool and on vesicles throughout the cytoplasm. Localization of myosin VI in NRK cells was confirmed further by expression of a myosin VI tail construct tagged with green fluorescent protein (GFP) at its N-terminus. The GFP-myosin VI tail is concentrated in the perinuclear region, partially co-localizing with TGN38 (Figure 1K, L and M). Deletion mutants have shown that just the globular tail alone is sufficient for targeting to the Golgi complex, suggesting that the information for localization at this organelle resides in the sequence of this globular domain (Buss *et al.*, 2001).

Myosin VI is associated with the trans-Golgi network

Brefeldin A (BfA), a fungal metabolite that inhibits ARF function when added to cells, causes the release of

peripheral coat proteins from the Golgi into the cytosol and redistributes integral membrane proteins from the Golgi cisternae into the ER (Lippincott-Schwartz *et al.*, 1989; Ladinsky and Howell, 1992; Reaves and Banting, 1992). In many cell types, the action of BfA also results in the TGN fragmenting into vesicles, which concentrate around the microtubule-organizing centre (MTOC) and are observed by immunofluorescence as a characteristic spot (Ladinsky and HowelloHowe, 1992; Reaves and Banting, 1992). When BfA was added to NRK cells or NRK cells transiently overexpressing the tail of myosin VI tagged with GFP, it caused endogenous myosin VI (Figure 2a) and the GFP-tail (Figure 2c) to coalesce into a spot which co-localized with the spot of TGN38 (Figure 2b and d) at the MTOC. This suggests that myosin VI is not associated with an ARF-dependent peripheral coat protein nor with a Golgi cisternal protein, but rather with an integral membrane protein at the *trans*-side of the Golgi complex at or close to the TGN.

To study more precisely the localization of myosin VI at the *trans*-side of the Golgi complex, electron microscopy (EM) sections of NRK cells were labelled with a monoclonal antibody to myosin VI tail and a polyclonal antibody to TGN38. As well as some myosin VI being present in a cytosolic pool, most myosin VI was localized to vesicular structures on the *trans*-side of the Golgi complex slightly away from the main TGN38 labelling (Figure 3A). We also 'quantified' the localization following immuno-EM of NRK cells stably overexpressing the globular tail of myosin VI tagged with GFP, using antibodies to GFP and to TGN38. The distance and direction of GFP-myosin VI globular tails to the closest labelled TGN38 molecule were also measured. The highest amount of GFP-myosin VI globular tail was found in the region 200–400 nm from the closest labelled TGN38 molecule in the direction away from and on the *trans*-side of the Golgi stack. GFP labelling was associated mostly with small vesicles (Figure 3B). These vesicles are not part of the recycling compartment, as in a transferrin uptake experiment myosin VI does not co-localize with transferrin at the ultrastructural level (data not shown).

Myosin VI is a peripheral membrane protein at the Golgi complex

Golgi membranes were prepared from rat liver by a standard procedure (Slusarewicz *et al.*, 1994). On SDS-polyacrylamide gels, a large number of proteins were present in these Golgi membrane preparations (Figure 4A, a). When probed with a polyclonal antibody raised against the whole tail of myosin VI, a single band of 140 kDa, the predicted size of myosin VI, was detected in these rat liver Golgi membrane preparations (Figure 4A, b). The inclusion of an additional incubation step with 1 M KCl in the protocol, and an extra centrifugation step in 1 M KCl led to the production of salt-washed Golgi membranes from which peripheral membrane proteins were stripped off. Immunoblotting salt-washed Golgi membranes shows that, when compared with the conventional Golgi membrane preparation, most of the myosin VI was removed (Figure 4A, c), implying that myosin VI is a peripheral membrane protein in the Golgi complex.

Fig. 1. Myosin VI is present in the Golgi complex. Localization of endogenous myosin VI in NRK cells was detected by immunofluorescence microscopy using a range of different polyclonal and monoclonal antibodies: (A) a polyclonal antibody to the C-terminal globular tail domain (poly a-GT), (D) a monoclonal antibody to the whole tail (mono a-tail) and (G) a polyclonal antibody to the whole tail (poly a-tail). In addition, myosin VI tail tagged with GFP was overexpressed transiently in NRK cells (K). The Golgi complex was visualized with either a monoclonal (B, H and L) or polyclonal (E) antibody to TGN38. The merged images are shown in (C), (F), (I) and (M). All of the myosin VI antibodies used labelled myosin VI in the Golgi complex in addition to myosin VI elsewhere in the cell. Bar = 5 μ m.

There are two pools of myosin VI around the Golgi complex

Purified Golgi membrane preparations contain actin filaments. To establish whether myosin VI was bound to the actin filaments or to a receptor/binding protein on the Golgi membranes, the membranes were extracted with either a detergent or with Mg-ATP, or both together. Treatment with Mg-ATP releases myosin VI from actin filaments if it is bound via its motor domain, and there is an 8- to 10-fold reduction in the amount of myosin VI bound to actin filaments when cell extracts are prepared in the presence of Mg-ATP (Buss *et al.*, 1998). Extraction of

Golgi membranes with Triton X-100 and Mg-ATP at 200 mM NaCl released all of the associated myosin VI into the soluble fraction (Figure 4B, g). In the control lane when neither Triton X-100 nor ATP was added, all of the myosin VI remained associated with the Golgi membranes (d) whereas, when either Triton X-100 (e) or ATP (f) were added, about half of the total amount of myosin VI is released into the supernatant. From these results, we conclude that there are two separate pools of myosin VI associated with the Golgi complex: one sensitive to ATP and therefore probably bound to actin filaments via the motor domain, and another one sensitive to detergent

extraction and therefore most probably bound to a receptor protein present on Golgi membranes via its tail domain.

The lack of myosin VI causes changes in Golgi morphology

In order to study the role of myosin VI in Golgi morphology and function, we prepared skin fibroblasts from Snell's waltzer mice lacking myosin VI. A cross of two heterozygous mice provided a litter with two homozygous wild-type mice, four heterozygous mice and two homozygous Snell's waltzer mice. Primary fibroblasts from each mouse were prepared and cultured for several weeks. Fibroblasts from each littermate were immunoblotted with a polyclonal antibody to the tail of myosin VI. Whereas approximately equal amounts of myosin VI were expressed in wild-type and heterozygous mice, no myosin VI protein was detectable in Snell's waltzer mice (Figure 5A). Immunoblots for myosin II and actin were used to show that approximately equal amounts of protein had been loaded onto the gel.

Interestingly, immunofluorescence studies on the mouse fibroblasts showed a noticeable difference in the appearance of the Golgi complex. In general, the Golgi complexes in the Snell's waltzer cells were smaller and more fragmented, compared with the wild-type Golgi complexes which were more extensive and reticulate throughout the cell. These significant differences in the

Golgi complexes were seen using antibodies to two Golgi marker proteins, TGN38 and GM130 (Figure 5B). We quantitated this initial observation by measuring the area of the Golgi complex and the area of the cell, in order to calculate the percentage area occupied by the Golgi

Fig. 2. Redistribution of myosin VI after brefeldin A (BfA) treatment. To visualize the effect of BfA on the subcellular localization of myosin VI, NRK cells were grown for 2 h in the presence of 5 μ g/ml BfA. Endogenous myosin VI was detected using a polyclonal antibody to the whole tail (a) and overexpressed GFP myosin VI tail by GFP fluorescence (c). The position of the rearranged TGN in the 'spot' close to the MTOC was visualized using a monoclonal antibody to TGN38 (b and d). Bar = 5 μ m.

Fig. 3. Immuno-EM localization of myosin VI close to the TGN. (A) Two representative micrographs showing immuno-EM localization of myosin VI close to the TGN. Cryosections from NRK cells were labelled with a mouse monoclonal antibody to myosin VI and a rabbit polyclonal antibody to TGN38, followed by goat anti-mouse immunoglobulin (15 nm) and goat anti-rabbit immunoglobulin (5 nm). Bar = 200 nm. (B) Quantitation of immuno-EM labelling of stable NRK cell lines expressing GFP-myosin VI globular tail. Distances were measured between 5 nm labelled TGN38 molecules and the closest 15 nm labelled myosin VI globular tail-GFP molecules. Data from 45 separate Golgi complexes were compiled.

Fig. 4. Myosin VI is a peripheral membrane protein present in two pools around the Golgi complex. **(A)** Myosin VI is present in purified Golgi membranes. Rat liver Golgi membranes were separated by SDS-PAGE and stained with Coomassie blue (a). Immunoblotting of the Golgi membranes (b) and of the salt-washed Golgi membranes (c) with antibodies to the whole tail of myosin VI. Most of the myosin VI was removed upon salt washing, suggesting that it is a peripheral membrane protein. **(B)** Extraction of purified Golgi membranes using Triton X-100 and/or ATP. Without Triton X-100 or ATP, all of the myosin VI remained in the pellet (p) (d). When either Triton X-100 or ATP was added to the Golgi membranes (e and f), approximately half of the myosin VI associated with the Golgi membranes was released into the supernatant (s). When both Triton X-100 and ATP were added to the Golgi membranes (g), all of the myosin VI was released into the supernatant. The appearance of a second myosin VI band when the membranes were treated with Triton X-100 and/or ATP occurs only sometimes, and we are unable to provide a satisfactory explanation other than assigning it to degradation.

complex in the Snell's waltzer and wild-type cells. This was repeated for a statistically robust sample size of 100 primary cells from each of two wild-type, two heterozygous and two homozygous Snell's waltzer mice. These results were confirmed on primary fibroblasts from homozygous Snell's waltzer mice, and homozygous and heterozygous wild-type mice from a second litter (data not shown). Furthermore, the same results were also obtained from measurements made on immortal cell lines (prepared following the protocol described in Materials and methods; data not shown). A statistical test (t -test) was performed to confirm the significant difference ($P < 0.001$) in the area of the cell occupied by the Golgi complexes; the area occupied by the Golgi complex was on average 40% less in Snell's waltzer cells (Figure 5C). There was no statistically significant difference in Golgi size between fibroblasts from homozygous (wt/wt) and heterozygous (wt/sv), i.e. phenotypically wild-type mice, which express similar levels of myosin VI when compared by immunoblotting. Confocal Z-sectioning was also carried out to

Fig. 5. Absence of myosin VI leads to an alteration in Golgi morphology. **(A)** Fibroblasts derived from homozygous (sv), heterozygous (h) and wild-type mice (wt) originating from one litter were used for immunoblotting with the antibody to the whole tail of myosin VI. Myosin VI was not detectable in Snell's waltzer fibroblasts (sv). To confirm equal loading of cell homogenates onto the SDS-polyacrylamide gel, it was blotted with antibodies to myosin II and actin. **(B)** In Snell's waltzer cells, Golgi complex morphology was changed. Snell's waltzer and wild-type cells were stained by immunofluorescence with a polyclonal antibody to TGN38 (a and b). The Golgi complexes in Snell's waltzer cells appeared much smaller and more fragmented than those in wild-type cells, which generally appeared larger and more reticulate. This observation was also seen by staining with GM130, a marker protein of the Golgi stack (c and d). Bar = 5 μ m. These initial observations were confirmed by quantification of the Golgi area (stained with antibodies to TGN38) in relation to the cell size (C). This calculation was performed on primary cells from two wild-type mice (wt), two heterozygous mice (h) and two homozygous Snell's waltzer mice (sv). Error bars: $\pm$ SEM. There was an ~40% reduction in Golgi size in Snell's waltzer cells.

measure the approximate thickness of Golgi complexes. The average thicknesses from sample groups were 1.3 μ m for the Golgis in Snell's waltzer fibroblasts and 1.8 μ m for those in wild-type fibroblasts. This represents a statistic-

Fig. 6. Golgi morphology can be rescued by transfection of whole myosin VI. (A) GFP–whole myosin VI and GFP–myosin VI globular tail (GT) were transfected into Snell's waltzer cells and the Golgi complex visualized by staining with antibodies to TGN38. Both whole myosin VI (c) and the globular tail (a) are targeted to the Golgi complex and to vesicular structures. This Golgi staining shows partial overlap with TGN38 (b and d). Bar = 5 μ m. (B) Measurement of the area occupied by the Golgi complex in these transiently transfected cells shows that transfection of whole fully functional myosin VI (MVI) was able to rescue the reduced Golgi complex morphology in the Snell's waltzer cells to the wild-type size. Transfection of only the globular tail (GT) did not rescue Golgi morphology. UT = untransfected Snell's waltzer cells. Error bars: $\pm$ SEM.

ally significant difference, and means that since both the area and the height of the Golgi complex are reduced in Snell's waltzer fibroblasts, the overall volume is also decreased. At the ultrastructural level, no obvious differences in Golgi morphology between wild-type and Snell's waltzer fibroblasts such as vesiculation or loss of stacking were observed (data not shown).

Transfection of whole myosin VI into Snell's waltzer cells can rescue Golgi morphology

In order to test whether we could restore wild-type Golgi morphology in Snell's waltzer fibroblasts, rescue experiments were attempted. For these experiments, either whole functional myosin VI or only the globular tail of myosin VI, both tagged with GFP, were transfected and expressed transiently in Snell's waltzer fibroblasts (Figure 6A). Both constructs, as expected, were targeted to the Golgi complex (Figure 6A, a and c) and found in clathrin-coated vesicles (data not shown). Transfected cells were co-stained with TGN38 (Figure 6A) or GM130 (data not shown), and the Golgi size was measured as described

Fig. 7. Constitutive secretion is reduced in Snell's waltzer cells. (A) In order to measure constitutive secretion, a construct which expresses a soluble secreted form of the alkaline phosphatase was transfected into Snell's waltzer and wild-type fibroblasts. The amount of alkaline phosphatase secreted into the tissue culture supernatant was measured 48, 72 and 96 h after transfection and plotted as a percentage of total maximal secretion in wild-type cells after 96 h. Transfection efficiency was normalized by co-transfection of a control plasmid expressing GFP. Secretion in Snell's waltzer cells was reduced by ~40–50% compared with wild-type levels. (B) Rescue experiments were performed by generating Snell's waltzer cell lines stably expressing only GFP (a), the globular tail of myosin VI (b) or whole myosin VI (c). Levels of secretion were compared with wild-type cells stably expressing only GFP (d). Only Snell's waltzer cell lines stably expressing whole myosin VI show levels of secretion for alkaline phosphatase similar to wild-type levels. Error bars: $\pm$ SD.

above. This quantitation was performed for a statistically relevant sample size of 100 transfected cells for each construct. Transfection with only the globular tail of myosin VI had no effect on the reduced size and morphology of the Golgi complex in Snell's waltzer cells. Transfection of whole fully functional myosin VI, however, was able to rescue Golgi size and morphology in Snell's waltzer cells back to wild-type levels (Figure 6B).

The lack of myosin VI in Snell's waltzer fibroblasts leads to reduced secretion

Newly synthesized proteins and lipids pass through and are sorted in the Golgi complex on their way to the cell surface or to endosomal compartments. In order to measure constitutive secretion in Snell's waltzer cells, they were transfected with a plasmid encoding a secreted form of alkaline phosphatase (SEAP) (Towler *et al.*, 2000). Newly synthesized enzyme is transported into the lumen of the ER from where it trafficks constitutively through the Golgi complex and then to the cell surface,

Fig. 8. Myosin VI co-localizes with Golgi-derived cargo vesicles. NRK cells were transiently transfected with the temperature-sensitive construct VSVG tagged with GFP. The cells were incubated at 39.5°C overnight, then moved to 19.5°C the following day for 2 h, and then to 32°C for 50 min. The last temperature shift causes release of the VSVG protein from the Golgi complex, and it is trafficked towards the plasma membrane. The cells were fixed after 50 min at 32°C, and stained with a polyclonal antibody against myosin VI. VSVG can be seen to co-localize with myosin VI at the Golgi complex, and also in vesicular structures surrounding the Golgi complex. Bar = 5 μ m.

where it is released into the medium. Measuring the amounts of alkaline phosphatase present in the cell culture medium up to 4 days after transfection indicated that there was a 40% reduction in secretion of this enzyme (Figure 7A) in Snell's waltzer immortal fibroblastic cell lines as compared with wild-type. In order to perform rescue experiments, immortal Snell's waltzer fibroblastic cell lines were stably transfected with GFP, the globular tail of myosin VI tagged with GFP or whole myosin VI tagged with GFP. In these rescue experiments, only fully functional myosin VI was able to restore wild-type levels of secretion. Expression of either myosin VI tail or GFP alone was not able to increase the levels of secretion (Figure 7B). To examine the synthesis of SEAP, we also lysed fibroblasts to measure internal levels, and we found that intracellular levels are very similar in wild-type and Snell's waltzer fibroblasts, suggesting that once a certain critical level of AP has been produced in the cell, it is either secreted or degraded.

Myosin VI is present on VSVG-containing cargo vesicles

The temperature-sensitive VSVG construct tagged with GFP was transfected into NRK cells, and trapped in the ER

overnight by incubation at 39.5°C. Subsequent transfer to 19.5°C caused the accumulation of the protein in the Golgi complex, and then movement to 32°C enabled the protein to exit the Golgi complex, and be trafficked to the plasma membrane. Cells were fixed and stained with an antibody against myosin VI after 50 min at 32°C. Myosin VI can be seen to co-localize with VSVG-GFP in the Golgi complex itself, and also in vesicular structures that have left the TGN. This co-localization, highlighted by the arrows in Figure 8A–F, suggests that myosin VI might indeed be involved in moving cargo vesicles away from the Golgi complex.

Discussion

Immunofluorescence microscopy using antibodies raised against myosin VI tail and the use of myosin VI tail tagged with GFP have both clearly shown that myosin VI is localized at the Golgi complex (Figure 1). Myosin VI is also present in vesicular structures partially co-localizing with clathrin-coated vesicles, in membrane ruffles and as a free cytosolic pool (Buss *et al.*, 1998, 2001). The monoclonal antibody showed the clearest Golgi staining, whereas the polyclonal antibodies to the globular tail or

the whole tail also recognized myosin VI at its different locations throughout the cell, presumably due to selective accessibility of different epitopes. In double labelling experiments, all three antibodies showed partial co-localization with TGN38, and immuno-EM showed that both intact myosin VI and stably expressed myosin VI tail-GFP are concentrated mostly in vesicles on the *trans*-side of the Golgi complex, with a concentration of these vesicles 200–400 nm away from the TGN.

When rat liver Golgi membranes were immunoblotted (Figure 4A), the presence of myosin VI in the Golgi complex was also revealed. Biochemical manipulation using high salt concentrations (Figure 4A, c) showed that myosin VI is a peripheral membrane protein of the Golgi complex. It probably binds to another protein resident in the Golgi complex, since there are no obvious consensus sequences in the tail of myosin VI suggesting direct binding to membranes. The use of a combination of salt, detergent and Mg-ATP treatment (Figure 4B) showed that myosin VI is present on membranes of the Golgi complex and also on the actin filaments surrounding the complex. Since only half of the myosin VI can be released with Mg-ATP (which releases myosin heads from actin) from purified Golgi membrane preparations, it indicates that the remaining half of myosin VI must bind directly to a Golgi protein, probably via its globular tail. Therefore, the myosin VI present in purified Golgi membrane preparations cannot be regarded as just a contaminant binding to co-purified actin filaments.

Having established the presence of myosin VI in the Golgi complex, we then proceeded to study the role of this molecular motor in the function of this organelle. The Snell's waltzer mouse provides an excellent system for studying the effects of the absence of myosin VI on the Golgi complex. These mice lack any myosin VI protein due to an intragenic deletion in the region of the gene encoding the motor domain. Their most obvious phenotype is deafness, but defects in other organs and tissues have not yet been studied in detail. Using fibroblasts derived from these mice (Figure 5), we have shown that the Golgi complexes are altered significantly in morphology and reduced in size, compared with fibroblasts from a wild-type background (Figure 5). Endocytosis, as measured by transferrin uptake and TGN38 antibody uptake experiments, was comparable in Snell's waltzer and wild-type fibroblastic mouse cells (data not shown). However, levels of secretion from the TGN to the plasma membrane were reduced by ~40% in Snell's waltzer cells compared with the wild-type levels (Figure 7). The lack of effect of the absence of myosin VI on clathrin-mediated endocytosis in fibroblastic cells was not a surprising since wild-type fibroblasts lack the myosin VI isoform linked to this process (Buss *et al.*, 2001).

Transfection of a myosin VI tail construct into Snell's waltzer mouse cells had no effect on the Golgi complex, but transfection of whole fully functional myosin VI led to complete rescue of the altered Golgi complex morphology (Figure 6) and to a rescue of the reduced secretion levels to virtually wild-type levels (Figure 7). Based on these results, we propose that myosin VI may have dual roles in the Golgi complex; it may play a functional role by being involved in vesicle trafficking by moving cargo away from the Golgi complex, and it may also play a structural role in

maintaining Golgi complex morphology. The challenge now is to elucidate the exact way in which myosin VI plays these two roles.

Myosin VI is an actin-based molecular motor which moves towards the minus ends of actin filaments (Wells *et al.*, 1999). Most other members of the myosin superfamily, with the exception of myosin IX (Inoue *et al.*, 2002), move towards the plus end of actin filaments. Therefore, to suggest a possible role for myosin VI at the Golgi complex, knowledge of the orientation of actin filaments in the cell is of utmost importance. At the plasma membrane, the orientation of the actin filaments is such that the plus ends abut the membrane, leaving the minus ends pointing into the centre of the cell. Little is known about the orientation of actin filaments on intracellular organelles, but work on phagosomes has suggested that the plus ends abut the membrane of the organelle, with the minus ends pointing into the cytosol (Defaque *et al.*, 2000). A speculative extension of these observations would be that the actin filaments surrounding the Golgi complex have their plus ends towards the membrane and their minus ends pointing away. This would implicate myosin VI, the minus end-directed motor, in movement away from the Golgi complex.

So what roles is myosin VI likely to play at the Golgi complex? There are several possibilities; since we know that a meshwork of actin filaments surrounds this organelle, myosin VI may be involved in transporting cargo short distances on actin filaments away from the Golgi complex to microtubules for longer distance transport through the cell (Toomre *et al.*, 1999; Kreitzer *et al.*, 2000). This possible function is supported by our EM data highlighting a concentration of myosin VI tail-GFP in vesicles in close proximity to the TGN. Myosin VI may also be involved in the actual physical budding process of vesicles from the TGN, or it could be involved at an earlier step such as deformation of the TGN membrane. Furthermore, myosin VI could be involved in sorting of newly synthesized proteins and lipids in the TGN for their different destinations inside the cell. At the plasma membrane, a newly identified binding partner for myosin VI is Dab2, a linker molecule binding to the cytoplasmic tail of members of the low-density lipoprotein receptor family (Morris *et al.*, 2002). Myosin VI therefore is implicated directly in clathrin-mediated uptake of this type of receptor into the cell, particularly at the apical surface of polarized cells with microvilli. Whether by analogy myosin VI at the TGN is associated via different linker molecules to specific cargo in the Golgi complex is not known. The localization of myosin VI at the Golgi complex and with membrane ruffles at the leading edge in motile fibroblasts might suggest that it is involved in sorting of cargo to be delivered to sites of ruffling. In a resting, non-motile fibroblast, exocytic vesicles arrive all over the cell surface. In a migrating fibroblast, however, newly synthesized proteins are now exported to the leading edge in a Rab8-dependent manner (Peraenen *et al.*, 1996). This directed transport to a specialized surface of the fibroblast is accompanied by actin and microtubule rearrangements possibly causing positioning of the Golgi complex on the side of the nucleus closest to the leading edge. Myosin VI could therefore play a role in sorting to the leading edge.

Myosin VI also appears to play a role in maintaining Golgi complex morphology based on our observations of a reduction in size and alteration in morphology of the Golgi complex in cells lacking myosin VI. Again, we can only speculate about how myosin VI is able to maintain Golgi complex morphology (Seeman *et al.*, 2000): perhaps it anchors the actin filaments around the organelle to the membranes of the Golgi complex, or perhaps its movement away from the Golgi complex pulls the membranes outwards, keeping them taut. It has been reported previously (Valderrama *et al.*, 1998) that actin filaments are involved directly in the subcellular localization and morphology of the Golgi complex. Using cytochalasin D on tissue culture cells, it was shown that the Golgi complex collapses into a spot near to the centrosome region. In addition, it has been shown that morphological changes in the Golgi complex correlate with actin cytoskeletal rearrangements (Di Campli *et al.*, 1999). These results support our observations that the actin-based motor protein myosin VI is involved in maintaining Golgi morphology.

Myosin VI, however, except in the hair cells in the inner ear, appears to be a non-essential gene, since Snell's waltzer mice lacking myosin VI are able to survive. It is likely that redundant or alternative vesicle trafficking pathways are upregulated in the cells in these mice. There are now several examples where the lack of one particular protein in vesicle trafficking machinery (e.g. pearl mice which lack an AP-3 subunit; Zhen *et al.*, 1999) does not lead to death, because pathways act in concert with each other. Furthermore, depolymerization of actin filaments does not completely inhibit secretion in fibroblastic cells, it only slows it down. For a more dramatic effect of lack of myosin VI at the Golgi complex, instead of the fibroblastic cells used here, we need to look at cells such as pancreatic cells which are specialized for regulated secretion.

In summary, our data imply a function for myosin VI in vesicle transport away from the TGN, and in maintaining Golgi complex morphology.

Materials and methods

Antibodies

The monoclonal antibody to myosin VI (IB4) was generated from mice immunized with the whole tail of myosin VI, and the fusion reaction was carried out as described (Harlow and Lane, 1988). The antibody was used as undiluted tissue culture supernatant. The rabbit polyclonal antibodies to the whole tail and globular tail of myosin VI were prepared and affinity purified as previously described (Buss *et al.*, 1998). The rabbit polyclonal antibody to mouse TGN38 was a gift of Dr Matthew Seaman (Cambridge Institute for Medical Research, Cambridge, UK), and the mouse monoclonal antibody to rat TGN38 was from Dr G. Banting (University of Bristol, Bristol, UK). Other antibodies used in this study were: a mouse monoclonal antibody to GM130 (Transduction Laboratories), a rabbit polyclonal antibody to TGN38 (Luzio *et al.*, 1990), a mouse monoclonal antibody to GFP (Qbiogene, UK) and a rabbit polyclonal antibody to GFP (Molecular Probes, Leiden, The Netherlands). Gold-conjugated goat anti-rabbit immunoglobulin antibodies and goat anti-mouse immunoglobulin antibodies were from Biocell (UK).

Construction of GFP-myosin VI globular tail

Full-length (4 kb) chicken brush border myosin VI cDNA (GenBank accession No. AJ278608) (Buss *et al.*, 1998), the whole tail (amino acids 846–1277) or only the globular tail (amino acids 1036–1273) of myosin VI were cloned into the mammalian expression vector pEGFP (Clontech, Basingstoke, UK) so that they were expressed with GFP at their N-terminus (Buss *et al.*, 2001). Stable cell lines expressing GFP-myosin

VI or deletion fragments were established using Δ pMEP as the expression vector (Girotti and Banting, 1996).

Immunofluorescence and immuno-EM

NRK cells were obtained from the European Collection of Animal Cell Cultures and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Immunofluorescence staining was performed as described in Buss *et al.* (1998).

BFA (Sigma, UK) was used at a concentration of 5 μ g/ml, and cells were incubated with the drug for 2 h prior to being fixed.

Immuno-EM on frozen ultrathin sections was performed as described by Griffiths (1993) and Reaves *et al.* (1996). Control NRK cells and NRK cells stably overexpressing the globular tail of myosin VI tagged with GFP were fixed with 8% formaldehyde in 0.1 M Na-cacodylate buffer for 1 h at room temperature, embedded in 15% polyvinylpyrrolidone/2.3 M sucrose and sectioned using a Leica UCT ultra microtome. The cryosections were labelled with primary antibodies to TGN38, myosin VI and GFP as appropriate, followed by gold-conjugated secondary antibodies. The sections were then contrasted with 0.3% uranyl acetate/methylcellulose and viewed under a Phillips CM100 transmission electron microscope. Measurement of distance between gold particles was performed using an onboard measurement facility (Analysis, Germany).

Transfection of cells

For transient transfection experiments, NRK cells, primary Snell's waltzer or wild-type mouse cells were grown on coverslips to ~70% confluence and transfected transiently according to the manufacturer's instructions with 2 μ g of pEGFP-myosin VI (whole or tail deletion mutant) using FuGENE™ (Roche Diagnostics, UK). At 24 h post-transfection, the cells were fixed and treated as above for the immunofluorescence experiments. For selection of stable cell lines, Snell's waltzer mouse immortal cell lines were transfected as described above with whole myosin VI-GFP, the globular tail-GFP or only GFP cloned into Δ pMEP. To be able to grow wild-type immortal mouse cells under the same selecting conditions, cells were transfected with only GFP in Δ pMEP. Selection was performed by addition of 200 μ g/ml of hygromycin B (Roche Diagnostics, UK) to the culture medium. Expression of GFP-tagged protein was induced by addition of 5 μ M CdCl₂.

NRK cells were transiently transfected with the VSVG-GFP-N1 construct (Clontech, Palo Alto) using FuGENE™ as above, and the cells were incubated at 39.5°C overnight. The next day the cells were incubated at 19.5°C for 2 h, and then shifted to 32°C for 50 min. At this time, the cells were fixed and stained with a polyclonal antibody to myosin VI by the immunofluorescence protocol described earlier.

Preparation of rat liver Golgi membranes

Rat liver Golgi membranes were prepared essentially as described by Slusarewicz *et al.* (1994). Buffer A consisting of 0.1 M KPO₄ and 5 mM MgCl₂ was used to prepare five density gradient solutions containing 0, 0.25, 0.5, 0.86 and 1.0 M sucrose, respectively. Six livers from adult male Wistar rats were minced into small pieces in 0.5 M sucrose in buffer A. The tissue was homogenized by pressing it through a 150 μ m mesh steel sieve, added to six discontinuous sucrose gradients (prepared from the five density gradient solutions) between the 0.25 and 0.86 M layers and centrifuged in a swing-out rotor at 140 000 g for 1 h at 4°C. The Golgi fraction was collected from the 0.5 M–0.86 M interface and diluted to 0.25 M sucrose in buffer A. These membranes were underlaid with layers of 0.5 and 1 M sucrose in buffer A and centrifuged in a swing-out rotor at 140 000 g for 40 min at 4°C. The Golgi layer was collected from the 0.5 M–1 M interface, and the pooled Golgi fractions were diluted to 0.25 M sucrose, then centrifuged at 9000 g for 30 min at 4°C. The pellet was resuspended in a total of 3 ml of 0.25 M sucrose in buffer A.

Extraction of Golgi membranes

A buffer of 200 mM KCl, 10 mM HEPES, 1 mM dithiothreitol (DTT) and 10% glycerol was used to make up a 1% Triton X-100 solution, a 5 mM ATP/Mg²⁺ solution and a 1% Triton X-100 + 5 mM ATP/Mg²⁺ solution. Rat liver Golgi membranes were incubated in these solutions for 1 h at 4°C, and then centrifuged at 13 000 g. The supernatant was removed and precipitated with 30% (w/v) trichloroacetic acid (TCA). Both the pellet and supernatant were run on an 8% SDS-polyacrylamide gel, and immunoblotted with a polyclonal antibody to the tail of myosin VI, as described earlier.

Electrophoresis and immunoblotting

Electrophoresis of proteins and immunoblotting were carried out as described in Buss *et al.* (1998).

Establishment of primary cultures of mouse fibroblasts

A litter of newly born mice generated from a cross of two heterozygous *sv* mice (from the MRC Institute of Hearing Research in Nottingham) was genotyped using a PCR method as described in Self *et al.* (1999). Skin and muscle tissue (1 cm × 1 mm × 1 cm) from these newly born mice was minced in 1–2 ml of phosphate-buffered saline (PBS). The tissue pieces were incubated in 5× trypsin for 2 h at 37°C, then 6–8 ml of DMEM was added, and the large tissue lumps were disrupted by pipetting up and down. Single cells and tissue fragments were seeded in tissue culture flasks, and the cells were cultured over a period of several months in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 60 µM 2-mercaptoethanol. Spontaneous mutations in some cells led to immortalization of mouse fibroblasts. Using this protocol, two immortal cell lines from two different Snell's waltzer mice and two wild-type immortal cell lines from two different mice were generated.

Measurement of Golgi areas

The Golgi complexes in the mouse cells were stained with an antibody to TGN38 by the immunofluorescence protocol described earlier. The boundary of the Golgi complex and of the cell itself was drawn around using a drawing tool in the IP lab computer program (Scientific Imaging software, Scanalytics Inc., Fairfax, USA), and thus the percentage area of the cell occupied by the Golgi complex could be determined. This was repeated for >100 cells for each sample. The statistical test carried out to compare the wild-type and mutant areas was a two-tailed, unpaired *t*-test, with 95% confidence intervals. Confocal Z-sectioning measurements were taken using a Zeiss LSM 510 microscope, and analysed with the Zeiss LSM program.

Secreted alkaline phosphatase (SEAP) expression and assay

Snell's waltzer and wild-type mouse cells were transiently transfected with the pSEAP2-Control mammalian expression plasmid (Clontech, Palo Alto, CA) containing SEAP cDNA using FuGENE™ as described earlier. Transfection efficiency was normalized by co-transfection of a second plasmid expressing GFP. Extracellular media samples were taken every 24 h after transfection for 6 days, and 15 µl aliquots were assayed to determine the relative levels of SEAP secreted into the extracellular medium. SEAP enzyme assays were carried out using the chemiluminescent substrate CSPD [disodium 3-(4-methoxy-2-sulphato-1,2-dioxetane-3,2'-chloro)tricyclo decan-4-yl]phenyl phosphate] in a chemiluminescence assay (Clontech). Lysis was carried out with passive lysis buffer for 15 min at room temperature (Invitrogen). The assays were performed in 96-well flat-bottomed microtitre plates, and detection was with a plate luminometer (Berthold Detection Systems Orion Microplate Luminometer). Light signals were recorded as 10 s integrals.

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