Interaction of the actin cytoskeleton with microtubules regulates secretory organelle movement near the plasma membrane in human endothelial cells

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Summary

The role of cytoskeletal elements in regulating transport and docking steps that precede exocytosis of secretory organelles is not well understood. We have used Total Internal Reflection Fluorescence (TIRF) microscopy to visualize the three-dimensional motions of secretory organelles near the plasma membrane in living endothelial cells. Weibel-Palade bodies (WPb), the large tubular storage organelles for von Willebrand factor, were labelled with Rab27a-GFP. By contrast, green fluorescent protein (GFP)-tagged tissue-type plasminogen activator (tPA-GFP) labelled submicron vesicular organelles. Both populations of GFP-labelled organelles underwent stimulated exocytosis. The movement of these morphologically distinct organelles was measured within the evanescent field that penetrated the first 200 nm above the plasma membrane. WPb and tPA-GFP vesicles displayed long-range bidirectional motions and short-range diffusive-like motions. Rotating and oscillating WPb were also observed. TIRF microscopy enabled us to quantify the contribution of actin and microtubules and their associated motors to the organelle motions close to the plasma membrane. Long-range motions, as well as WPb rotations and oscillations, were microtubule- and kinesin-dependent. Disruption of the actin cytoskeleton and inhibition of myosin motors increased the number of long-range motions and, in the case of WPb, their velocity. The actin and microtubules had opposite effects on the mobility of organelles undergoing short-range motions. Actin reduced the mobility and range of motion of both WPb and tPA vesicles, whereas microtubules and kinesin motors increased the mobility of WPb. The results show that the dynamics of endothelial secretory organelles close to the plasma membrane are controlled by the opposing roles of the microtubule and actin cytoskeletal transport systems.

Movies available online

Key words: TIRF, Evanescent field, Intracellular organelle motility, tPA, Rab27a, Weibel-Palade bodies, Cytoskeleton, Exocytosis, Nanotechnology

Introduction

Microtubules, actin filaments and their associated molecular motors drive the movement of intracellular organelles. Interaction between the two transport systems seems necessary for the correct delivery of cellular cargoes such as pigment granules (Marks and Seabra, 2001), axoplasmic vesicles (Kuznetsoy et al., 1992), endoplasmic reticulum (ER) vesicles (Tabb et al., 1998), immature and mature secretory granules (Hirschberg et al., 1998; Rudolf et al., 2001), phagosomes (Al-Haddad et al., 2001), lysosomes (Cordonnier et al., 2001), caveolar membranes (Mundy et al., 2002), postendocytic vesicles (Maples et al., 1997) or internalized virus particles (Pelkmans et al., 2002; Rietdorf et al., 2001). Several models have been proposed to account for the interaction of actin- and microtubule-based transport systems. In ‘sequential’ models, fast long-range transport is mediated by microtubules and actin provides short-range local transport or restricts the organelle movement at its final destination (Goode et al., 2000; Rogers and Gelfand, 2000; Wu et al., 2000). By contrast, ‘tug-of-war’ models assume that different types of motors act simultaneously and that the motion of an organelle results from a balance of the forces exerted on the organelle (Gross et al., 2002a; Gross et al., 2002b).

In the case of secretory organelles, the final destination is the plasma membrane, where the organelles undergo exocytosis. Although the cytoskeleton is involved in late transport steps and subsequent docking to and fusion with the plasma membrane, most studies have focused on the role of the actin cortex, which is thought to act as a barrier preventing organelle motion and docking to the plasma membrane (Valentijn et al., 1999). Less is understood about the role of microtubules and their interaction with the actin cytoskeleton to regulate the transport and movement of secretory organelles close to the plasma membrane.
Endothelial cells secrete by exocytosis several proteins that regulate blood coagulation, blood flow and local immune responses. The fibrinolytic tissue-type plasminogen activator (tPA) is found in small (0.1-0.2 μm diameter) secretory vesicles (Emeis et al., 1997; Schick et al., 2001). The pro-inflammatory adhesive protein von Willebrand factor (vWF) is stored in unique large tubular organelles (1-3 μm long, 0.1-0.2 μm diameter) called Weibel-Palade bodies (WPb) (van Mourik et al., 2002; Weibel and Palade, 1964). Disruption of microtubules blocks the regulated secretion of both tPA (Santell et al., 1992) and vWF (Sinha and Wagner, 1987; Vischer et al., 2000), suggesting that the microtubule cytoskeleton plays a central role in the processing and/or transport of tPA and vWF. WPb are members of the family of lysosome-related organelles, which includes the pigment granules of melanocytes (Marks and Seabra, 2001). Studies in melanocytes indicate that pigment granules are transported via microtubules to the cell periphery where they are trapped into the actin cytoskeleton through the myosin Va-receptor Rab27a (Hammer, III and Wu, 2002; Seabra et al., 2002). However, unlike pigment granules in melanocytes, nothing is known about the role of microtubules and the actin cytoskeleton in regulating secretory organelle dynamics in endothelial cells.

In this study, we have visualized WPb and tPA-containing vesicles in living endothelial cells by the expression of Rab27a-GFP (green fluorescent protein) and tPA-GFP, respectively. We have used Total Internal Reflection Fluorescence (TIRF) microscopy for three-dimensional single-particle tracking of fluorescent WPb and tPA vesicles, to quantify the movement of these morphologically distinct organelles and to study the role of the cytoskeleton and molecular motors in their transport and dynamics close to the plasma membrane.

Materials and Methods

GFP constructs

The Rab27a-GFP construct was a gift from M. Seabra (Hume et al., 2001). The tPA-GFP construct was obtained as follows. The full coding sequence of human tPA was generated by a PCR-based strategy using the plasmid pETPFR (ATCC) as a template and the coding sequence of human tPA was generated by a PCR-based strategy using the plasmid pETPFR (ATCC) as a template and the oligonucleotide primers 5'-gggaattcaatgcatggatgcaatgaagaggg-3' and 5'-cgcggtaccgctccggtcgcatgttgtcacgaatc-3'. The resulting DNA was ligated into the vector pEGFP-N3 (Clontech) that had been digested previously with EcoRI and KpnI.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were either isolated and grown as previously described (Carter et al., 1988) or purchased as cryopreserved cells from pooled donors (ZHC-2101, TCS CellWorks, Bucks, UK). No significant difference was observed between cells from the two different sources. Custom cell chambers were assembled by gluing a Teflon® frame to a glass slide. Cells were directly plated on the chambers without any additional coating and cultured at 37°C in a 5% CO₂ atmosphere. Freshly isolated cells were grown in M199 medium supplemented with 10% fetal calf serum (FCS), 10% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cryopreserved cells were grown in endothelial growth medium (ZHM-2953, TCS CellWorks).

Organelles and cytoskeleton labelling

Cells were microinjected with Rab27a-GFP or tPA-GFP cDNAs. Rab27a-GFP cells were imaged live 48 hours after microinjection. tPA-GFP-expressing cells were imaged live 4 hours after microinjection. Cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X100 and 10% FCS for 10 minutes before staining. For microtubule labelling, 0.5% glutaraldehyde (Sigma) was added during fixation. Primary antibodies were: rabbit polyclonal anti-human vWF diluted 1/100 (A0082, Dako, Denmark) and rat monoclonal anti-α-tubulin diluted 1/200 (clone YL1/2 MCAP77; Serotec, Oxford, UK). Secondary antibodies were Rhodamine (TRITC)-conjugated donkey anti-rat, Rhodamine (TRITC)-conjugated donkey anti-rabbit, Cy2-conjugated donkey anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA). Actin was stained with rhodamin-phalloidin (Sigma) diluted 1/500 for 45 minutes. Cells were left in PBS for TIRF imaging. For colocalization experiments, cells were fixed immediately after live imaging to minimize cell motion, cytoskeleton deformation and fixation artefacts. A secretory organelle localized with a cytoskeletal element when its fluorescence overlapped or was within one pixel from the corresponding cytoskeletal structure.

Drug treatments

The microtubule cytoskeleton was depolymerized by incubating cells in culture medium supplemented with 10 μM nocodazole (Sigma) for 1 hour at 37°C, 5% CO₂. The cell shape and adhesion to the glass slide remained satisfactory (data not shown). Nocodazole treatment selectively disrupted the microtubule network but left the actin cytoskeleton intact (data not shown). Microtubules repolymerized in 15 minutes after cells were washed twice in culture medium. Actin filaments were depolymerized using 5 μM latrunculin B (Calbiochem) for 10 minutes at 37°C, 5% CO₂. Under these conditions, few actin bundles remained visible in TIRF images, whereas microtubules were not significantly affected (data not shown). At higher concentrations or longer incubation times, dramatic shape changes and loss of adhesion of the cell to the glass substrate occurred. Kinesin motors were blocked with 10 μM arachidonic acid (AFA, Sigma) for 2 hours, whereas myosin motors were inhibited using 10 mM 2,3-butadieone monoxime (BDM, Sigma) for 30 minutes.

Dual-colour TIRF microscopy

Total internal reflection (reviewed by Axelrod, 2001; Styer and Almers, 2001; Toomre and Manstein, 2001) was achieved at the glass slide/culture medium interface using a trapezoidal glass prism. The refractive index of the prism and culture medium was n=1.52 and n=culture medium=1.336, respectively, giving a critical angle (θc) for total internal reflection of θc=61.5°. Experiments were carried out on an upright microscope (Axioskop, Zeiss, Oberkochen, Germany) using either an Argon ion laser (excitation λ=488 nm, 25 mW, Melles Griot, Carlsbad, CA) or a Nd:YAG laser (excitation λ=532 nm, 50 mW, CrystaLaser, Reno, NV). The angle of incidence of the excitation light could be adjusted and was fixed to 68-70°, above the critical angle. The intensity profile of the evanescent wave is exponentially decaying: I(z)=I0exp(−z/dp), where z is the vertical distance, I0=I(z=0) is the intensity at the interface and dp is the penetration depth given by:

\[ dp = \frac{\lambda}{4\pi n^2 \sin^2(\theta) - n^2} \]

The calculated penetration depth for the Argon ion laser and for the Nd:YAG laser was dp=75-85 nm and dp=85-95 nm, respectively.

GFP (respectively rhodamine) fluorescence was excited by the Argon ion (resp. Nd:YAG) laser. Differences in beam radius and divergence were corrected by additional lenses to achieve the same spot size and position on the sample. Light from TIRF images was passed through a dichroic filter (505DRLP02 for GFP and Cy2...
fluorescence or 560DRLP for rhodamine fluorescence, Omega Optical, VT) and an emission filter (530DF30 or 565ALP). Standard epifluorescence was achieved using a 100 W mercury lamp (excitation filters 485DF22 or 525DF45). The temperature in the cell chamber was maintained at 35-37°C by circulating temperature-controlled water around the prism. 5% CO2 in O2 was blown on the cell chamber through a collar around the objective.

Image acquisition
The light was collected with a 100× 1.0 NA water immersion objective (Zeiss) driven by a piezo-electric focus drive (Physik Instrumente, Waldbronn, Germany). Fluorescence images were magnified by a 0.5-2x optical zoom (Zeiss), processed by an Argus 20 image processor (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and collected with an intensified CCD camera (Remote Head Darkstar, S25 Intensifier, Photonics Science, UK). Images were digitized and stored in the memory of a Pentium III PC computer at a maximum rate of 25 frames/second by a frame grabber (IC-PCI 4Mb (AMVS), Imaging Technology, MA) and then saved to disk. Image processing was carried out using Optimas 6.5 (Media Cybernetics L. P., Silver Spring, USA). Live time-lapse images of GFP-labelled organelles were acquired at 0.5-2 frames/second with online two frames averaging performed by the Argus-20 image processor. TIRF illumination was limited to 200 frames to minimize photobleaching and phototoxicity. Dual-colour images of fixed cells were acquired with 256 frames averaging. The pixel size was 0.1092 μm (resp. 0.0870 μm) with a 1.6x (resp. 2x) optical zoom. The image size was typically 520x500 pixels.

Three-dimensional organelle tracking
Customized macros were written in the C++ based language ALI (Analytical Language for Images) and run under Optimas 6.5. The raw images (N frames, separated by the time interval δt) were filtered to enhance the visibility of the organelles. A low-pass Fast Fourier Transform filter followed by a 3x3 pixels trimmed mean filter was applied to remove nonuniform background. At the beginning of the sequence, a region of interest (ROI) containing the organelle and a threshold above which the organelle could be detected was defined. The ‘centre of grey’ (centre of mass weighted by pixel intensities) of the organelle was detected and the fluorescence intensity of the organelle Ipar measured was computed for the corresponding raw image in a 5x5 pixels box centered on the centre of grey. The local background fluorescence intensity Ibg was measured in another ROI close to the previous one but containing no organelle. The 2D position of the centre of grey yield the x and y coordinates of the organelles, whereas the z coordinate (relative to the initial position and corrected for local background brightness variations) was given by:

\[ z(t) = -dp \ln \frac{I_{\text{par}}(t) - I_{\text{bg}}(t)}{I_{\text{par}}(0) - I_{\text{bg}}(0)} . \]

Long-range directed motions and short-range diffusive-like motions
When visualized under the TIRF microscope, organelles displayed two types of motions: long-range directed motions and short-range diffusive-like motions. Long-range motions were defined as being >1 μm in distance and with a maximum velocity >0.05 μm/second. All other motions were considered as short-range motions. Long-range motions were identified by thresholding and accumulating the images from a time-lapse sequence (e.g. Fig. 3A) or using frame to frame subtraction. Averaging an entire sequence enabled static organelles undergoing short-range diffusive motions to be identified (e.g. Fig. 4A).

Data analysis
From the three-dimensional coordinates \( x(t), y(t), z(t) \) of the organelle, the mean squared displacement (MSD) travelled by the organelle during a time interval \( \Delta t = n \delta t \) was calculated according to:

\[ \text{MSD}(\Delta t = n \delta t) = \frac{1}{N-n} \sum_{j=1}^{N-n} \left[ (x(j \delta t + n \delta t) - x(j \delta t))^2 + (y(j \delta t + n \delta t) - y(j \delta t))^2 + (z(j \delta t + n \delta t) - z(j \delta t))^2 \right]. \]

The analysis of the plot of the MSD as a function of the time interval \( \Delta t \) allowed us to define three classes of motion (Steyer and Almers, 1999): simple diffusion (MSD(\Delta t) is a straight line), slow directed motion superimposed on diffusion (MSD(\Delta t) deviates from a straight line and shows an upward curvature), restricted diffusion in a ‘cage’ (MSD(\Delta t) shows a downward curvature). To quantify the motion of the vesicles, we used the following equations to fit the MSD data (Steyer and Almers, 1999): simple diffusion with a diffusion coefficient \( D \) was fitted with:

\[ \text{MSD}(\Delta t) = 6D \Delta t + \text{cst}; \]  

(1)
directed diffusion at velocity \( v \) and with a diffusion coefficient \( D \) was fitted with:

\[ \text{MSD}(\Delta t) = 6D \Delta t + v^2 \Delta t^2 + \text{cst}; \]  

(2)
and restricted diffusion was fitted using:

\[ \text{MSD}(\Delta t) = R^2 \left[ 1 - a_1 \exp(-a_2 D \Delta t/R^2) \right] + 6D_{\text{cage}} \Delta t + \text{cst}, \]  

(3)
where \( R \) is the radius of the cage, \( D_{\text{cage}} \) is the diffusion coefficient of the cage, \( D \) is the diffusion coefficient of the vesicle inside the cage, and \( a_1=0.99, a_2>0.85 \) are two constants. In Eqs 1-3, \( \text{cst} \) is a numerical constant accounting for the limited accuracy of the tracking algorithms. Eqs 1-3 are valid only at small enough time intervals, \( \Delta t \).

Because the \( x-y \) spatial resolution of the microscope (approx. 0.1 μm) is much lower than the vertical \( z \) resolution (approx. 0.01 μm), three-dimensional MSD data are dominated by 2D \( x-y \) motions. To specifically quantify displacements in the \( z \) direction, the vertical MSD was calculated according to:

\[ \text{MSD}_z(\Delta t = n \delta t) = \frac{1}{N-n} \sum_{j=1}^{N-n} \left[ (z(j \delta t + n \delta t) - z(j \delta t))^2 \right]. \]

The \( z \)-direction ‘diffusion coefficient’ was defined as \( 2D_z = \lim_{\Delta t \to 0} \text{MSD}(\Delta t) \) (Johns et al., 2001).

The resolution of the TIRF microscope was estimated by tracking 0.1 μm diameter polystyrene beads (Molecular Probes, Eugene, OR) stuck to a glass slide in the same conditions as when cells were observed. The signal-to-noise ratio in the bead images was adjusted to match that in the organelle images. Motions of the immobilized beads were due to mechanical vibrations in the experimental set-up. The minimum detectable 3D diffusion coefficient was \( D_z = 2.6 \pm 0.1 \times 10^{-6} \) μm²/second (\( n=10 \)), whereas the corresponding \( z \)-direction ‘diffusion coefficient’ was \( D_z = 1.2 \pm 0.2 \times 10^{-7} \) μm²/second (\( n=10 \)).

Fusion of secretory organelles with the plasma membrane
Secretion was stimulated by 100 μM histamine (Sigma). Cells were imaged at 2 frames/second before and during stimulation. Individual fusion events were analyzed by fitting a Gaussian distribution to the
fluorescence image. The diffusion coefficient of the fluorophore $D_{\text{fluo}}$ was deduced from a linear fit of the distribution half-width $R_{\text{fluo}}$ with time according to:

$$R_{\text{fluo}}^2(t) = 4D_{\text{fluo}} t + \text{cst}.$$ 

**Results**

**Visualization of secretory organelles close to the plasma membrane in living HUVECs**

Expression of a Rab27a-GFP chimera (Hume et al., 2001) fluorescently labelled Weibel-Palade bodies, the large rod-shaped secretory organelles of vWF storage in endothelial cells (Fig. 1C) [see article in this issue (Hannah et al., 2003)]. By contrast, tPA-GFP localized to small-diameter vesicles. With conventional epifluorescence excitation, the fluorescent tPA-containing and WPb organelles were mostly unresolved because of background fluorescence from regions of the cell such as the Golgi and the ER. However, the sectioning effect of TIRF microscopy enabled us to clearly visualize both populations of organelles within 200 nm close to the plasma membrane (tPA, Fig. 1A; WPb, Fig. 1B).

Fusion of Rab27a-GFP-positive organelles appeared as a fluorescent cloud spreading away from the site of fusion after 100 μM histamine stimulation (Fig. 2A). Fitting the half-width $R_{\text{fluo}}$ of the fluorescence distribution with time (Fig. 2C) gives the value for the diffusion coefficient of Rab27a-GFP in the plasma membrane: $D_{\text{fluo}}=0.24\pm0.16 \, \mu\text{m}^2/\text{second}$ ($n=3$). By contrast, exocytosis of tPA vesicles was observed as a transient brightening of the vesicle followed by an exponential decay of its fluorescence intensity (Fig. 2B,D) with a characteristic time $\tau = 12.3\pm1.6$ seconds ($n=13$). Fusion of tPA vesicles was more frequent ($n=29$ in eight cells) than fusion of Rab27a organelles ($n=3$ in ten cells).

**WPb and tPA vesicle movements in resting HUVECs**

Two types of motion were distinguished for WPb and tPA vesicles in resting HUVECs: long-range directed motions and much slower short-range diffusive-like motions (see Materials and Methods). Examples of time-lapse sequences are given in Supplementary Material (see Movies 1, 2; http://jcs.biologists.org/supplemental/).

**Long-range directed motions**

Approximately 22% of WPb within the evanescent field exhibited long-range motions, corresponding to a frequency, defined as the number of long-range motions per total number of organelles per unit time, of $1.0\times10^{-3}$ per second. Long-range motions often involved rapid changes or reversals in direction, changes in velocity and pauses between consecutive runs. The average velocity during a long-range run was $0.16\pm0.02 \, \mu\text{m}/\text{second}$ and the maximum velocity was $0.54\pm0.04 \, \mu\text{m}/\text{second}$ ($n=54$). Run length and run duration averaged $7.2\pm0.8 \, \mu\text{m}$ and $70.5\pm10.1 \, \text{seconds}$ ($n=54$) respectively. A reduction in WPb velocity often correlated with an increase in the fluorescence intensity of the organelle, indicating that it was moving closer towards the plasma membrane, as seen in Fig. 3B, where both sets of data are plotted in time. During pauses between directed runs, we observed complex behaviours of WPb, including rotations and oscillations (see Movies 3, 4; http://jcs.biologists.org/supplemental/). Rotations (e.g. Fig. 3C) occurred in either clockwise or anticlockwise directions with the same average frequency $\omega_{\text{rot}}=0.268\pm0.036 \, \text{rad/second}$ ($n=5$). In some cases, WPb exchanged their leading end without reversing the direction of their motion, probably as a result of combined rotation and translation. Oscillations (Fig. 3D) occurred preferentially during pauses and before direction reversals. The average oscillation frequency was $\omega_{\text{osc}}=0.141\pm0.005 \, \text{per second}$ ($n=6$).

TPA vesicles also exhibited long-range motions. The frequency of long runs was greater than for WPb ($2.2\times10^{-3}$ per second), as were the average velocity, maximum velocity and...
average run length [0.67±0.07 µm/second, 1.48±0.13 µm/second and 8.2±1.1 µm (n=35), respectively]. As with WPb, the velocity of tPA vesicles often decreased when moving closer towards the plasma membrane (not shown).

**Short-range diffusive-like motions**

The majority (>70%) of WPb close to the plasma membrane undergo diffusive-like short-range motions (e.g. Fig. 4A). Plots of the three-dimensional MSD as a function of the time interval $\Delta t$, revealed three classes of diffusive-like behaviours: simple diffusion, directed diffusion and restricted diffusion, occurring with approximately equal frequency. MSD($\Delta t$) plots for individual WPb (Fig. 4A) were fitted according to Eqns 1-3 (see Materials and Methods). The diffusion coefficient $D$, drift velocity $v$ (directed diffusion), cage radius $R_{cage}$ and diffusion coefficient $D_{cage}$ (restricted diffusion) derived from these fits are summarized in Table 1. The averaged MSD plots are shown in Fig. 4B.

Similar results and orders of magnitude were obtained for tPA vesicles, except that in the case of caged diffusion, the cage was about five times more mobile than for WPb (data not shown).

**Localization of the cytoskeleton and secretory organelles close to the plasma membrane of HUVECs**

In fixed cells, microtubules and actin filaments were found to extend into the region illuminated by the evanescent field, coming closest in proximity to the plasma membrane in peripheral regions of the cell (Fig. 5). Actin formed stress fibres with enhanced fluorescence at sites of focal adhesion contacts (Fig. 5A). A more diffuse actin staining was also visible over the cell footprint and was probably due to a more random organization of short filaments in the actin cortex. Microtubules were generally oriented radially, with tubulin fluorescence often greater on peripheral microtubules, indicating that they come closer to the plasma membrane in these regions (Fig. 5B). Quantification of dual-colour TIRF images (see Materials and Methods) showed that 58.1±3.2% of WPb colocalized with microtubules (n=15 cells) and 28.5±2.7% colocalized with actin fibres (n=14 cells). Similar results were obtained for tPA-GFP vesicles (data not shown). WPb clearly aligned with microtubules, particularly at the cell periphery near microtubule plus-ends (Fig. 5C).

**Table 1. Characteristics of Weibel-Palade bodies undergoing short-range motions in non-treated cells**

| Class              | Proportion (%) | Diffusion coefficient $D$ (µm$^2$/s) | Diffusion coefficient $D_{cage}$ (restricted diffusion) (µm$^2$/s) | Cage radius $R_{cage}$ (nm) |
|--------------------|----------------|---------------------------------------|-------------------------------------------------------------------|-----------------------------|
| Simple diffusion   | 44.3±3.0%      | 3.4±0.7 $10^{-4}$                      |                                                                   |                             |
| Directed diffusion | 23.3±2.7%      | 1.9±0.9 $10^{-4}$                      |                                                                   |                             |
| Restricted diffusion| 32.4±5.6%     | 3.9±0.7 $10^{-3}$                      |                                                                   | 187±16 nm                  |

The averaged parameters deduced by fitting the three-dimensional MSD plots according to Eqns 1-3 are shown for each class of diffusive behaviour (n=21, 11 and 16 for simple, directed and restricted diffusion respectively).
Superposition of movies of WPb movements on images of the microtubule system after fixation indicates that long-range motions of WPb frequently occur along microtubules (Movies 5A,B; http://jcs.biologists.org/supplemental).

Long-range dynamics depend primarily on the microtubule cytoskeleton

Microtubule disruption caused a significant decrease in the density (number of organelles per \( \mu m^2 \)) of both populations of secretory organelles (WPb: 16.9±7.0\%, \( n=14 \); tPA vesicles: 39.8±14.7\%, \( n=4 \)), suggesting a role for the microtubule cytoskeleton in the transport of both types of organelles to the cell periphery. Disruption of microtubules almost completely abolished long-range motions of WPb (Fig. 6A). In nocodazole-treated cells, the frequency of WPb undergoing long-range motions dropped from 1.0×10^{-3} to 8×10^{-5} per second (Fig. 6B). For the few remaining WPb undergoing long-range motion, the total displacement was just above the arbitrary threshold for the definition of long-range motion, and the average and maximum velocities of WPb were four times slower than in control cells (Fig. 6B). Complex dynamics such as oscillations and rotations seen in control cells were not observed in nocodazole-treated cells. Long-range directed motions, oscillations and rotations reappeared when microtubules repolymerized after nocodazole washout (data not shown).

In the presence of the kinesin ATPase inhibitor aurintricarboxylic acid (ATA) (Hopkins et al., 2000), long-range motions were unidirectional and mostly directed towards the cell centre (78.6\%, \( n=14 \)). Both the average and maximum velocities significantly increased (\( P<0.04 \)), whereas the total run length decreased (Fig. 6B).

Disruption of actin filaments and inhibition of myosin motors by 2,3-butanedione monoxime (BDM) (Cramer and Mitchison, 1995) had more subtle effects. Actin depolymerization did not induce any significant change in organelle density for either WPb (4.1±3.0\%, \( n=4 \)) or tPA vesicles (4.7±13.7\%, \( n=4 \)). Latrunculin B and BDM treatments induced an increase in the frequency of long-range motions of WPb and a decrease in the total run length (Fig. 6B). The average velocity increased significantly (\( P<0.04 \)) on actin disruption and myosin inhibition (Fig. 6B).

Qualitatively similar results were obtained for tPA-GFP vesicles, except that the average velocity in latrunculin B-treated cells was the same as in control cells (data not shown). Taken together, these results indicate that long-range motions are essentially microtubule-dependent, and that actin

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**Fig. 3.** Long-range movements of WPb in HUVECs. (A) Long-range directed motions visualized by thresholding and accumulating frames from a sequence of 120 images. Bar, 10 \( \mu m \). (B) Example of long-range directed motion. The three-dimensional trajectory is shown in the left-hand panels. Note the difference in scale between the \( z \)-axis and the \( x-y \) axes. The initial point (\( t=0 \)) is indicated by an asterisk. The right-hand panel shows plots of vertical position \( z \) (solid line, left axis in \( \mu m \)) and velocity (dotted line, right axis in \( \mu m/second \)) as a function of time, with a rolling average of three frames. The WPb slows down as it approaches the plasma membrane (arrows) and accelerates as it moves away from the membrane (arrowheads). (C) WPb rotation. Plot of the angle of a rotating WPb as a function of time (anticlockwise rotation, frequency \( \omega_{rot}=0.40 \) per second). Top panels show the corresponding TIRF images (Bar, 1 \( \mu m \)). (D) WPb oscillations. Plot of the \( x-y \) velocity of an oscillating WPb as a function of time (oscillation frequency \( \omega_{osc}=0.14 \) per second).
decreases their frequency and, in the case of WPb, their velocity.

Short-range diffusive motions are controlled by actin and microtubule elements

Short-range motions of WPb were affected by actin and microtubule depolymerization and by inhibition of kinesin and myosin motors (Figs 7, 8). We pooled data from all three classes of diffusive-like behaviours (simple, directed and restricted diffusion) (Fig. 7). On treatment with nocodazole, the average diffusion coefficient decreased by a factor 4.3, whereas it increased by a factor of 4.2 with latrunculin B treatment (Fig. 7A). Effects of cytoskeleton disruption also showed up on the averaged 3D MSD plots (obtained by averaging all MSD plots together, as shown on Fig. 7A, right panel). Blocking kinesin motors activity by ATA induced a decrease in the diffusion coefficient by a factor 2.7. By contrast, treatment with the myosin inhibitor BDM did not change the value of the diffusion coefficient. Drug treatments had qualitatively similar effects on the vertical

Fig. 4. Short-range motions of WPb in HUVECs. (A) Short-range diffusive motions visualized by averaging the same sequence as in Fig. 3A. Examples are shown of simple diffusion (WPb 1), directed diffusion (WPb 2) and restricted diffusion (WPb 3). The WPb x-y trajectories are given in the centre panels (x and y in μm). Three-dimensional MSD plots (right-hand panels) were fitted according to Eqs 1-3 (see Materials and Methods). The parameters deduced from the fits are: WPb 1, $D = 1.5 \times 10^{-4}$ μm$^2$/second; WPb 2, $D = 1.05 \times 10^{-3}$ μm$^2$/second, $v = 1.07 \times 10^{-2}$ μm/second; WPb 3, $D = 1.0 \times 10^{-4}$ μm$^2$/second, $D_{cage} = 3.6 \times 10^{-5}$ μm$^2$/second, $R_{cage} = 49$ nm. Bar, 10 μm. (B) Averages of simple diffusion ($n = 21$), directed diffusion ($n = 11$), and restricted diffusion ($n = 16$) 3D MSD plots.

Fig. 5. Visualization of cytoskeletal elements in HUVECs by TIRF microscopy. (A) TIRF image of the actin cytoskeleton labelled with rhodamine-phalloidin. Arrowheads point to focal adhesions. The diffuse staining is probably due to the actin cortex. (B) TIRF images of the microtubule cytoskeleton visualized by immunostaining of α-tubulin. Left-hand panel: cell with radially oriented microtubules; arrowheads show bright peripheral microtubules. Right-hand panel: detail of the cell periphery in another cell. (C) WPb align with microtubules. Arrowheads indicate WPb colocalizing with microtubules in two different cells. WPb preferentially accumulate at microtubule plus-ends (green, Rab27a-GFP; red, α-tubulin). Bars, 10 μm.
diffusion coefficient $D_z$. The $z$-direction averaged MSD$_z$ plots from nocodazole- or latrunculin B-treated cells also clearly differed from control cells (Fig. 7B, right panel).

The distribution and characteristics of WPb diffusive motions in each class of diffusive-like behaviours (simple, directed and restricted diffusion) were affected by the disruption of microtubules or actin filaments (Fig. 8). Nocodazole treatment almost completely abolished restricted diffusive behaviours, and the proportion of directed diffusion increased compared with simple diffusion (Fig. 8A). The drift velocity $v$ of directed diffusive motions did not change (not shown), whereas the diffusion coefficient of the cage ($D_{\text{cage}}$) and the cage radius ($R_{\text{cage}}$) strongly decreased (Fig. 8B). Latrunculin B treatment decreased the proportion of restricted diffusion in favour of simple diffusion. The drift velocity $v$ increased by a factor of 2.5 (not shown), and the cage radius significantly increased ($P<0.04$) (Fig. 8B). Kinesin and myosin inhibition by ATA and BDM, respectively, did not significantly affect the distribution of diffusive motions among the three classes (Fig. 8A).

The effects of cytoskeleton disruption on the short-range motions of tPA vesicles differed in two respects from those on WPb movements (data not shown). Nocodazole treatment did not modify the average value of the diffusion coefficient and latrunculin B treatment slightly decreased the drift velocity of directed diffusion of tPA vesicles.

### Discussion

**Transport and exocytosis of WPb and tPA vesicles in living endothelial cells visualized by TIRF microscopy**

We have used TIRF microscopy to investigate the dynamics of fluorescent Weibel-Palade bodies and tPA vesicles close to the plasma membrane and their localization with respect to cytoskeletal elements. Expression of Rab27a-GFP produced fluorescent rod-like organelles, morphologically indistinguishable from native WPb, that were colabelled with a specific antibody to vWF, the major WPb protein (Fig. 1C). Consistently, endogenous Rab27a also localizes to the WPb membrane (Hannah et al., 2003). Expression of tPA-GFP in HUVECs produced small fluorescent organelles similar to those seen in untransfected cells (Emeis et al., 1997; Schick et al., 2001; Zupancic et al., 2002). Both Rab27a-GFP and tPA-GFP organelles undergo stimulated exocytosis, as shown by individual fusion events with the plasma membrane on histamine stimulation (Fig. 2). After exocytosis, Rab27a-GFP diffuses in the plasma membrane with a diffusion coefficient of $0.24\pm0.16 \, \mu m^2/second$, in agreement with values reported previously for other membrane-associated proteins (Saxton and Jacobson, 1997). By contrast, tPA-GFP brightened on fusion and then slowly dimmed at the fusion site for a characteristic time of $12.3\pm1.6$ s. This may be due to resealing and reacidification of the vesicles after exocytosis, as reported recently in PC-12 cells (Taraska et al., 2003).
Secretory organelle transport visualized by TIRF microscopy

Immunohistochemical localization of WPb or tPA vesicles revealed a close association with microtubules (Fig. 5C and data not shown), and disruption of microtubules but not of the actin cytoskeleton decreased the density of WPb and tPA vesicles in close proximity to the plasma membrane. This indicates that a proportion of these organelles are transported to and/or maintained at the cell periphery via microtubule-dependent processes.

Actin interacts with microtubules during long-range transport of WPb and tPA vesicles

WPb were seen to move continuously over distances of up to 20 μm (mean run length approx. 7 μm). Long-range motions were saltatory and bidirectional, with velocities in the range of 0.1-1.0 μm/second. These characteristics are compatible with microtubule-driven motility observed in vitro and in vivo (Howard, 2001). This was confirmed by the almost complete loss of long-range motions when microtubules were disrupted by nocodazole (Fig. 6). Moreover, nonspecific inhibition of kinesin motors with the kinesin ATPase inhibitor aurantricarboxylic acid (ATA) (Hopkins et al., 2000) strongly decreased the frequency of long-range motions of WPb (Fig. 6B). Microtubule-dependent transport of WPb to the plasma membrane is consistent with the observation that microtubule disruption blocks vWF secretion (Sinha and Wagner, 1987; Vischer et al., 2000).

tPA vesicles moved three to four times faster than WPb and their run lengths were, on average, longer by 1 μm. The motors responsible for the motion of tPA vesicles and WPb may be different. However, another hypothesis is that collective behaviours of motors control the motility of large organelles (Welte et al., 1998). Long-range transport of WPb may thus be slower due to pools of microtubule motors with opposite polarity being active at the same time. Indeed, blocking kinesin activity increased the velocity of WPb (Fig. 6B), suggesting that competition with dynein motors slows down motion on microtubules. The presence of multiple motors may also give rise to the rotations and oscillations displayed by WPb (Fig. 3C,D). Oscillations frequently occurred during pauses or before direction reversals. Bidirectionality and oscillations disappeared when kinesin ATPase activity was blocked.

Actin and actin-based motors also play a role in the long-range motility of WPb and tPA vesicles. Long-range motions

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**Fig. 7.** Effects of cytoskeleton disruption on short-range motions of WPb. (A) Data from all classes of short-range diffusive behaviours (simple, directed and restricted diffusion) were pooled to calculate the average diffusion coefficient $D$ (left panel) and to plot the averaged three-dimensional MSD (right panel) in nontreated cells (circles or NT, $n=48$ from three cells), nocodazole-treated cells (diamonds or noco, $n=51$ from three cells), cells treated with the kinesin inhibitor ATA ($n=48$ from three cells), latrunculin B-treated cells (triangles or latB, $n=60$ from three cells) and cells treated with the myosin inhibitor BDM ($n=60$ from three cells). A numerical constant was added to the averaged MSD data so that all three plots coincide on their first data point. (B) Same analysis as in A for vertical motions (in the $z$ direction) in nontreated cells ($n=47$ from three cells), nocodazole-treated cells ($n=46$ from three cells), cells treated with the kinesin inhibitor ATA ($n=41$ from three cells), latrunculin B-treated cells ($n=53$ from three cells) and cells treated with the myosin inhibitor BDM ($n=53$ from three cells). The left-hand panel shows the $z$-direction diffusion coefficient $D_z$. Averaged one-dimensional MSD, are plotted on the right panel. A numerical constant was added to the averaged MSD data so that all three plots coincide on their first data point.
WPb and tPA vesicles was of the order of about 100 nm diameter bead diffusing in the cytoplasm, as found for diffusive motions of chromaffin granules in chromaffin cells (Han et al., 1999; Lang et al., 2000). By comparison, a chromaffin cell (as also found for diffusive motions of chromaffin granules in diffusive-like motions (Fig. 4). The diffusion coefficient of the organelles to microtubules via molecular motors. This is restricting short-range movements of WPb and tPA vesicles near the plasma membrane. About 80% of WPb and tPA vesicles close to the plasma membrane. The actin cytoskeleton and microtubules play opposing roles in short-range motions of WPb and tPA vesicles close to the plasma membrane.

The actin cytoskeleton and microtubules play opposing roles in short-range motions of WPb and tPA vesicles close to the plasma membrane.

Restriction of short-range movements of WPb and tPA vesicles near the plasma membrane

About 80% of WPb and tPA vesicles within the evanescent field appeared to be almost immobile, undergoing short-range diffusive-like motions (Fig. 4). The diffusion coefficient $D$ for WPb and tPA vesicles was of the order of $D=10^{-4}$ $\mu$m$^2$/second, as also found for diffusive motions of chromaffin granules in chromaffin cells ($D=10^{-4}$-10$^{-2}$ $\mu$m$^2$/second) (Oheim et al., 1999; Steyer and Almers, 1999; Steyer et al., 1997), and PC12 cells (Han et al., 1999; Lang et al., 2000). By comparison, a 100 nm diameter bead diffusing in the cytoplasm would have a diffusion coefficient 1000 times larger ($D=kT/(6\pi\eta R)=10^{-1}$ $\mu$m$^2$/second; taking the cytoplasm viscosity $\eta =6\eta_{\text{water}}=6 \times 10^{-3}$ kg/m/s). Thus, diffusive motions of secretory organelles close to the plasma membrane are severely restricted. The secretory organelles may be docked to the plasma membrane or bound to cytoskeletal elements via complexes of molecular motors. Their motion could also be physically but nonspecifically hindered by the cortical actin gel.

The diffusion coefficient of the 'cage' and the 'cage' radius for both organelles increases on actin depolymerization (Fig. 8B). The proportion of restricted diffusion is also reduced compared with nontreated cells (Fig. 8A), further suggesting that actin not only decreases the mobility of the organelles but also physically restricts their range of motion. Restricted diffusion could thus represent motions within the cortical actin network (Lang et al., 2000), and the increase in ‘cage’ radius may reflect a widening of the network upon latrunculin B treatment. Instead of acting as a barrier preventing the docking of secretory organelles to the plasma membrane (Johns et al., 2001; Rudolf et al., 2001; Valentijn et al., 1999), the actin cortex could actually promote docking and fusion of the organelles with the plasma membrane by reducing their mobility and increasing the probability of association of complementary docking complexes. Interestingly, blocking myosin ATPase activity does not affect either the diffusion coefficient (Fig. 7) or the proportion of restricted diffusive motions compared with control cells (Fig. 8A), further suggesting that the cortical actin gel physically reduces organelle mobility at the plasma membrane.

On microtubule depolymerization, we observed a decrease in three-dimensional and vertical diffusion coefficients (Fig. 7). The proportion of restricted diffusion behaviour is also strongly reduced compared with nontreated cells (Fig. 8A), suggesting that restricted diffusion may be due not only to physical hindrance by the actin cortex but also to a tethering of the organelles to microtubules via molecular motors. This is also consistent with the decrease in organelle density observed.

Fig. 8. Effects of cytoskeleton disruption on simple, directed and restricted diffusive behaviours of WPb. A percentage of simple (S), directed (D) and restricted (R) diffusion in nontreated cells (NT), nocodazole-treated cells (noco) and latrunculin-treated cells (latB).

(A) Simple diffusion. Proportion of simple (S), directed (D) and restricted (R) diffusion.

(B) Restricted diffusion. Diffusion coefficient of the cage ($D_{\text{cage}}$ in $\mu$m$^2$/second) and radius of the cage ($R_{\text{cage}}$ in $\mu$m) in nontreated cells (n=16), nocodazole-treated cells (n=1) and latrunculin-treated cells (n=11).
with nocodazole treatment. Interestingly, although actin plays a qualitatively similar role in the short-range mobility of tPA vesicles and WPb, we found that the role of microtubules was markedly different. The average diffusion coefficient of tPA vesicles was not modified when microtubules were depolymerized. As discussed above, a larger number of microtubule-based motors is probably recruited onto the surface of WPb, and this may increase their mobility. Consistent with this hypothesis, inhibition of kinesins by ATA reduces the average diffusion coefficient of WPb (Fig. 7) without affecting the distribution among the three classes of diffusive behaviours (Fig. 8A). These data show that microtubules increase the mobility of WPb near the plasma membrane, at least partly via the activity of kinesin motors.

Kinesin motors are supposed to act in the recruitment of secretory vesicles during a step preceding an actin-dependent step (Bi et al., 1997). Our results show that short-range dynamics of WPb near the plasma membrane are regulated not only by actin but also by microtubules and kinesin motors, indicating that microtubules could also play a role in later stages, such as organelle docking or fusion. Microtubules, probably via kinesin motors, counteract the confining effect of actin by increasing short-range mobility of WPb at the plasma membrane. Actin and microtubules may thus play opposing roles to fine-tune the mobility and localize organelles at target sites on the plasma membrane.

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